Analysis of Poplar (*Populus trichocarpa* × *P. deltoides*) Inducible Defense Response against Insect Herbivores

by

Ian Major
B.Sc., University of Alberta, 2000

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Abstract

In order to analyze the inducible defense response of hybrid poplar (*Populus trichocarpa* × *P. deltoides*), macroarrays were used to profile transcript patterns elicited by wounding and by regurgitant from forest tent caterpillar (FTC; *Malacosoma disstria*), a poplar defoliator. FTC regurgitant proved to be a potent elicitor of defense gene expression and was shown to contain the insect-derived elicitor volicitin. Comparison of inducible defense responses elicited by FTC regurgitant and wounding with pliers revealed qualitatively similar responses in terms of transcript accumulation. Extensive overlap was also observed in the sets of induced genes from locally- and systemically-induced leaves. Systemic responses were further investigated and shown to also be inducible in roots, which implies shoot-root systemic signaling. Comparative macroarray analysis showed similarities between inducible responses in leaves and roots, including genes that encode previously identified leaf herbivore defense genes.

The macroarray analysis also established a suite of marker genes for future studies of herbivore defense in poplar, many of which may play key roles in the defense response and are candidates for further study. Among these genes were several inducible Kunitz trypsin inhibitors (KTIs), which were investigated further with biochemical analyses. The sequenced poplar genome was used to select KTI genes that represent the diversity of this family. Recombinant proteins were generated and showed that the poplar KTI genes encode functional proteinase inhibitors and that they are functionally distinct, i.e. they

have specific proteinase substrate preferences. Moreover, wounding increases accumulation of KTI proteins, as well as protease inhibitor activity in leaves, supporting a defensive role for this protein family. These proteins were therefore tested for their ability to inhibit insect digestive proteases from FTC and bertha armyworm. The poplar KTIs tested all inhibited at least some protease activity from FTC midgut extracts. The strongest inhibitor of FTC proteases, TI3, was further tested in bioassays and shown to reduce larval growth of FTC when incorporated into insect diet, confirming that this KTI functions as an anti-herbivore protein. In addition, analysis of FTC midguts from TI3 feeding experiments showed that larvae responded to TI3 by producing more gut proteases. This hyperproduction of proteases may exacerbate the antinutritive effects of TI3 by reducing pools of essential amino acids.

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List of Abbreviations

AAE:	acyl-activating enzyme
ARG:	arginase
ATP:	adenosine triphosphate
BAW:	bertha armyworm
BCIP:	5-bromo-4-chloro-3-indoyl phosphate
BLAST:	basic local alignment search tool
BSA:	bovine serum albumin
BTEE:	benzoyl-L-tyrosine ethyl ester
cATPC:	chloroplastic ATP synthase γ-subunit
cDNA:	complementary DNA
DAB:	3,3'-diaminobenzidine tetrahydrochloride
dCTP:	deoxycytidine triphosphate
DNA:	deoxyribonucleic acid
DTT:	dithiothreitol
DUF:	domain of unknown function
eATP:	extracellular adenosine triphosphate
EDTA:	ethylenediamine tetraacetic acid
EST:	expressed sequence tag
EtBr:	ethidium bromide
FAC:	fatty acid-amino acid conjugate
FTC:	forest tent caterpillar
FTC-R:	forest tent caterpillar regurgitant
GOX:	glucose oxidase
GST:	glutathione-S-transferase
IC_{50}:	fifty-percent inhibitory concentration
IPTG:	isopropyl-β-D-thiogalactopyranoside
JA:	jasmonic acid
KTI:	Kunitz trypsin inhibitor
LC-MS:	liquid chromatography-mass spectrometry
LPI:	leaf plastochron index
MAP:	mitogen-activated protein
MJ:	methyl-jasmonate
mRNA:	messenger ribonucleic acid
MW:	molecular weight
NBT:	nitroblue tetrazolium chloride
NLS:	nuclear localization signal
OGA:	oligogalacturonic acid
P-value:	probability value
PBS:	phosphate buffered saline
PCR:	polymerase chain reaction
PI:	proteinase inhibitor
PM:	peritrophic membrane
PMSF:	phenylmethylsulfonyl fluoride

poly(A^+):	polyadenine
PPO:	polyphenol oxidase
PVDF:	polyvinylidene fluoride
Q-value:	false discovery rate
RNA:	ribonucleic acid
rRNA:	ribosomal ribonucleic acid
Rubisco:	ribulose-1,5-bisphosphate carboxylase/oxygenase
SBBI:	soybean Bowman-Birk inhibitor
SDS:	sodium dodecyl sulfate
SDS-PAGE:	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SSH:	suppression subtractive hybridization
SSPE:	saline sodium phosphate ethylenediamine tetraacetic acid
STI:	soybean Kunitz trypsin inhibitor
SucAla3pNA:	N-succinyl-Ala-Ala-Ala-p-nitroanilide
TAME:	p-toluene-sulfonyl-L-arginine methyl ester
TD:	threonine deaminase
TI:	trypsin inhibitor
TLCK:	N_α-tosyl-L-lysyl-chloromethane hydrochloride
TPCK:	N-*p*-tosyl-L-phenylalanine chloromethyl ketone
VSP:	vegetative storage protein
WIN:	wound-induced protein
ZIM:	zinc-finger protein expressed in inflorescence meristem

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1 Introduction

1.1 Overview of inducible plant defenses against insect herbivory

Plants respond rapidly to feeding by herbivore insects with active defensive mechanisms. These include physical barriers, such as trichomes and leaf toughness, and chemical defenses consisting of proteins and phytochemicals. Defenses can be present constitutively (developmentally regulated) or induced by the onset of herbivore feeding. Inducible protein and phytochemical defenses act as toxins, antifeedants, or antinutrients, and can directly reduce insect fitness (Walling, 2000; Gatehouse, 2002; Kessler and Baldwin, 2002). In addition, some responses induced by herbivory do not act directly against the feeding herbivore, but instead function by recruiting predators and parasitoids to the damaged plant, via the release of volatile compounds and production of extrafloral nectaries (Arimura et al., 2005). A key feature of many defense responses is their induction not only at the site of insect feeding, but also throughout the plant, which is known as the systemic response. Thus, when faced with herbivory, plants actively engage a complex defense response that ultimately renders the entire plant less susceptible to further consumption. The benefits of inducible defenses have been directly shown, as plants engineered to express higher levels of various herbivore defense genes exhibit enhanced resistance (Haq et al., 2004; Wang and Constabel, 2004a; Zavala and Baldwin, 2004; Chen et al., 2005). By contrast, plants in which the inducible defense response is silenced are often hypersusceptible to herbivore attack (Orozco-Cardenas et al., 1993; Kessler et al., 2004; Kang et al., 2006). It has been demonstrated that plant defense mechanisms have measurable fitness costs, and plants with high levels of defensive compounds exhibit reduced fitness in the absence of herbivore challenge (Baldwin, 1998; Kessler and Baldwin, 2002; Zavala et al., 2004). These fitness costs may have provided the selective pressures driving the evolution of inducible defenses (Kessler and Baldwin, 2002).

Seminal work by Green and Ryan (1972) first demonstrated inducible anti-herbivore defense in plants and systemic defense induction in undamaged tissues of damaged plants. Since then, extensive research has advanced our understanding of how insect attack is perceived by plants, how defense signaling proceeds and is translated into the defense response, and the identity of many defensive proteins and metabolites. A

major challenge is to understand how these compounds reduce damage caused by feeding insects. It is also now established that insects have evolved counter strategies to survive plant defenses (Gatehouse, 2002).

Much of what is known about inducible herbivore defense is based on studies of annual, herbaceous plants, and our knowledge of defense in woody perennials is much more limited. This thesis examines inducible defense of hybrid poplar (*Populus trichocarpa* × *P. deltoides*), which has become a model system for tree genomics and molecular biology. In this introductory chapter, I will describe our current understanding of inducible plant defense with examples of how plant defenses, especially proteins, reduce insect growth (see below). I will later describe the perception of herbivore damage (section 1.3), the subsequent plant-wide, systemic defense signaling (section 1.4), the use of gene expression profiling for studying plant defense responses (section 1.5), and finally the inducible defense response of the *Populus* genus (section 1.6).

1.2 Plant compounds with anti-insect properties

Numerous plant proteins are effective anti-herbivore agents (Constabel, 1999; Carlini and Grossi-de-Sa, 2002; Sadasivam and Thayumanavan, 2003). In general, plant defense proteins appear to utilize two defense strategies in herbivores guts: they can reduce the nutritive content of ingested plant tissues or act aggressively against herbivore digestive tissues. The following section discusses plant proteins that have been shown to directly contribute to resistance against insect herbivores.

1.2.1 Proteinase inhibitors

The introduction to proteinase inhibitors (PIs) described in this section will be brief, since they are discussed again in Chapters 5 and 6. PIs have been extensively studied for their effects on insect herbivores and are induced in response to wounding in many plants (Ryan, 1990). As their name implies, plant PIs inhibit proteolytic enzymes in the herbivore gut, and therefore prevent or reduce protein digestion. There are at least ten distinct families of PIs based on their amino acid sequences, and PIs that inhibit each of the four mechanistic classes of digestive proteinases have been identified (Laskowski and Kato, 1980; De Leo et al., 2002; Rawlings et al., 2004). Most plant PIs inhibit serine

proteinases, which is likely a reflection of the prevalence of these enzymes in herbivore digestive systems, since serine proteinases have been identified from many insect orders (Terra and Ferreira, 1994). Extensive research of PIs, including X-ray crystallography of inhibitors bound to target proteases, suggests a similar mechanism of inhibition for most plant PI families. Plant PIs are competitive inhibitors with a reactive site that binds tightly to the active groove of the cognate protease (Laskowski and Kato, 1980). Extensive intramolecular hydrogen bonds and disulfide bridges stabilize the PI and allow it to remain tightly bound to the protease even after hydrolysis. Plant PIs are a favorite target gene for engineered pest resistance through biotechnology, as numerous studies have reported increased resistance in transgenic plants overexpressing such genes (Haq et al., 2004). For example, serine PIs such as soybean Kunitz PI and cysteine PIs such as rice oryzacystatin have been used effectively against lepidopterans and coleopterans, respectively (Leplé et al., 1995; McManus et al., 1999).

Although PI inhibition of insect protease activity has been shown *in vitro* with gut extracts, when incorporated into artificial diets or expressed in plant tissue, PIs sometimes have little or no effect *in vivo* (Jongsma and Bolter, 1997). Subsequent analyses demonstrated that insects can adapt to the PI via hyperproduction of proteases to compensate for lost proteolytic activity (Broadway and Duffey, 1986), or by an adaptive switch of midgut proteases from PI-sensitive to PI-resistant proteases (Broadway, 1995; Jongsma et al., 1995). In some cases, the adapted PI-resistant proteases can also degrade the PIs, which not only detoxifies PIs but also uses them as a protein source (Michaud et al., 1995; Giri et al., 1998). By contrast, hyperproduction of proteases may deplete pools of essential amino acids in the herbivore and result in reduced insect performance (Markwick et al., 1998). The adaptation of insect digestive physiology to ingested PIs demonstrates that *in vitro* experiments do not always predict the *in vivo* effects of plant defenses.

1.2.2 Oxidative enzymes

Oxidative enzymes are suggested to act as antinutritive defenses by modifying or destroying essential nutrients in insect diets (Constabel, 1999). These enzymes include polyphenol oxidase (PPO), peroxidases, and lipoxygenases and their role in anti-

herbivore defense is supported by herbivore-induced expression (Thaler et al., 1996). PPOs catalyze the oxidation of monophenolic and *o*-diphenolic substrates to produce highly reactive *o*-quinones, which readily alkylate dietary proteins (Constabel, 1999). This prevents digestion and amino acid assimilation by insect herbivores and can profoundly reduce the nutritive value of insect diets (Duffey and Stout, 1996). The direct effects of PPO were recently shown with transgenic poplar overexpressing PPO. Larvae of forest tent caterpillar (FTC; *Malacosoma disstria*) exhibited significantly reduced growth and survival on poplar with elevated PPO levels compared with control plants (Wang and Constabel, 2004a). This was the first direct evidence for the anti-herbivore effects of PPO.

Peroxidases oxidize a wide variety of substrates and contribute to defense by reinforcing cell walls in addition to their antinutritive effects, which similar to PPO, involve oxidizing phenolics that results in alkylation of dietary proteins. Peroxidases contribute to lignification by generating phenoxy radicals which polymerize to form lignin (Douglas, 1996) and are likely also involved in cross-linking cell wall carbohydrates and proteins (Cassab and Varner, 1988). Lipoxygenases may directly act as antinutrients by destroying polyunsaturated fatty acids, which produces reactive fatty acid hydroperoxides (Duffey and Stout, 1996), and are also important for jasmonate synthesis (see section 1.4). Furthermore, the fatty acid hydroperoxide products react with dietary amino acids, destroying essential amino acids (Duffey and Felton, 1991). These effects have been shown to reduce the nutritive quality of dietary protein and the growth of corn earworm (*Helicoverpa zea*) (Felton et al., 1994). Ascorbic acid is a potent antioxidant and free radical scavenger and can therefore counteract the effects of the oxidative enzymes. Ascorbate oxidase is present at high levels in leaves of several plants species and may therefore enhance the effectiveness of the other oxidative enzymes by keeping pools of reduced ascorbate low (Duffey and Felton, 1991; Duffey and Stout, 1996).

1.2.3 Amino acid hydrolases

Recently, a novel proteomics-based approach was employed to identify tomato proteins that are stable (or undigested) in an insect herbivore's midgut and frass, and thus likely represent a suite of proteins with defense functions (Chen et al., 2005; Chen et al.,

2007). Among these stable proteins were arginase (ARG) and threonine deaminase (TD), which degrade the essential amino acids Arg and Thr, respectively. Both enzymes are active in tobacco hornworm (*Manduca sexta*) midguts, where increased ARG and TD activity is associated with reduced levels of free Arg and Thr (Chen et al., 2005). Furthermore, plants overexpressing ARG reduce larval growth. Additional analysis of tomato TD suggests that a specialized TD isozyme degrades Thr in insect midguts (Chen et al., 2007). The TD2 isozyme is hydrolyzed after insect ingestion which results in proteolytic removal of a C-terminal regulatory domain, allowing TD to efficiently deplete Thr in insect midguts without feedback inhibition. Moreover, TD-silenced *Nicotiana attenuata* plants are highly susceptible to *Manduca sexta* attack (Kang et al., 2006). It is intriguing that ARG and TD deplete essential amino acids in insect guts, since several other defense proteins, such as PIs and oxidative enzymes, reduce protein digestion in insect midguts. Together, the defensive roles of these proteins suggest that a major plant defense strategy is the limitation of essential amino acid assimilation, supporting theories that the protein quality of leaves can play a role in defense (Felton, 1996; Felton, 2005).

1.2.4 Proteases

Some defense proteins directly target and destroy structures within the insect midgut such as the peritrophic membrane (PM), which forms a physical barrier in insect guts and protect midgut cells from gut contents and pathogen attack. Specific proteases have been shown recently to be toxic due to their ability to attack insect PMs. The cysteine protease, maize Mir1, increases dramatically at the site of insect feeding, and fall armyworm (*Spodoptera frugiperda*) growth is reduced when reared on tissue expressing Mir1 (Pechan et al., 2000). Examination of the peritrophic matrix (PM) of larvae fed Mir1 by electron microscopy demonstrated that the PM was severely damaged, suggesting that the toxic effects of Mir1 result from disruption of the PM (Pechan et al., 2002). Recent *in vitro* experiments showed that dissected PMs treated with Mir1 are completely permeabilized, confirming that Mir1 directly disrupts the PM (Mohan et al., 2006). Mir1 also permeabilizes the PM of other Lepidopterans, including *Helicoverpa zea* and *Danaus plexippus*, although it does not affect the permeability of coleopteran and orthropteran insect families. The cysteine proteases papain, ficin and bromelain from the

latex of the papaya tree also reduce growth of lepidopteran larvae, including *Samia ricini*, *Mamestra brassicae* and *Spodoptera litura* (Konno et al., 2004). Moreover, the toxicity of papain, ficin and bromelain is dependent upon their protease activity, and they have also been shown to permeabilize the PM of *Spodoptera frugiperda* (Konno et al., 2004; Mohan et al., 2006).

1.2.5 Lectins

Lectins are carbohydrate-binding proteins that can have insecticidal activity against a diverse array of insect species (Vasconcelos and Oliveira, 2004). The insecticidal effects of specific lectins have been demonstrated with transgenic plants, as their overexpression in different plants increases resistance to insect attack (Carlini and Grossi-de-Sa, 2002). The precise insecticidal mechanism of lectins is not fully understood, but they appear to require the ability to bind to insect guts and resistance to proteolytic degradation in order to exert their deleterious effects. Some lectins bind to the brush-border membrane of insect gut epithelial cells or to the PM (Peumans and Van Damme, 1995). Also, some lectins can bind to glycosylated digestive enzymes. This binding may interfere with the digestive, protective or secretory functions of the midgut. For example, lectin from snowdrop (*Galanthus nivalis* agglutinin; GNA) decreases growth, development and survival of brown planthoppers (*Nilaparvata lugens*) (Powell et al., 1998). GNA is not degraded in the gut and binds to midgut epithelial cells, where it alters activity of midgut enzymes (Fitches and Gatehouse, 1998; Powell et al., 1998). The lectin was also found throughout the haemolymph of *Nilaparvata lugens*, suggesting that it can cross the midgut epithelium and thus potentially cause toxic effects systemically (Powell et al., 1998). Similarly, GNA accumulates in the guts and haemolymph of tomato moth (*Lacanobia oleracea*) (Fitches et al., 2001). Thus, lectins apparently exert their toxic effects via several mechanisms.

1.2.6 Chitinases

Chitinases are widespread among plants and are typically considered to be pathogen defenses (Kasprzewska, 2003). Since chitinases can be inducible by wounding or herbivory, they are sometimes suggested to defend against opportunistic wound

pathogens (Clarke et al., 1998). However, chitinases from some plants have anti-insect properties. In several cultivars of *Citrus*, increased chitinase activity in roots was associated with resistance to root weevil infestation (*Diaprepes abbreviatus*). Extracts of these roots were capable of degrading dissected weevil PM, suggesting a role for chitinase in insect defense (Mayer et al., 1995). Likewise, a chitinase purified from cowpea seeds (*Vigna unguiculata*) reduced the development of the cowpea weevil (*Callosobruchus maculatus*), presumably by acting on the insect PM (Gomes et al., 1996). Recently, the insecticidal effects of a poplar chitinase expressed in tomato leaves were demonstrated. Development of Colorado potato beetle (*Leptinotarsa decemlineata*) was significantly reduced when feeding on WIN6-expressing tomato leaves in two of three experiments (Lawrence and Novak, 2006). These studies suggested that the insecticidal activity of chitinase is due to degradation of the PM, but this has yet to be shown directly.

1.2.7 Other defense proteins

Several other proteins have been shown to have detrimental effects against insect herbivores. For example, a recent study demonstrated the insecticidal effects of an *Arabidopsis* acid phosphatase / vegetative storage protein (VSP). Plant VSPs have been identified in numerous plants and are sometimes induced by wounding or herbivory (Mason and Mullet, 1990; Davis et al., 1993), suggesting that these genes may play roles in plant defense against insects. Furthermore, *Arabidopsis VSP* expression levels are correlated with insect resistance (Berger et al., 2002). A recent functional analysis of recombinant *Arabidopsis* AtVSP2 protein confirmed predictions that *Arabidopsis* VSPs possess acid phosphatase activities (Liu et al., 2005). This AtVSP2 was tested for direct anti-insect activity in artificial diets and shown to reduce development and survival of two coleopterans (*Diabrotica undecimpunctata* and *Callosobruchus maculatus*) (Liu et al., 2005). Interestingly, mutagenesis of the acid phosphatase motif demonstrated that the acid phosphatase activity is responsible for the toxic effects of AtVSP2. Thus, plant acid phosphatases may also participate as plant defense proteins, although the mechanism of acid phosphatase toxicity remains to be determined.

1.2.8 Phytochemicals that function as anti-insect compounds

In addition to proteinaceous defenses, preformed and inducible phytochemicals, also known as secondary plant metabolites, are key components of herbivore resistance. Phytochemicals can perform a multitude of functions, which are generally associated with ecological processes. For example, they may function as attractants for pollination and seed dispersal, and protection from abiotic and biotic stresses. Phytochemicals that participate in anti-herbivore defense have a variety of mechanisms of action. Some deter insect herbivores, like the pungent odor of glucosinolates. Cyanogenic glycosides and glucosinolates are degraded by enzymes upon cellular decompartmentalization during insect feeding, releasing the toxins cyanide and isothiocyanate, respectively. Several volatile terpenoids are emitted in response to herbivory and attract predators and parasitoids of the feeding insects. Numerous reviews have discussed the anti-herbivore functions of phytochemicals such as phenolics, alkaloids, terpenoids, cyanogenic glycosides and glucosinolates in herbivore defense (Bennett and Wallsgrove, 1994; Zagrobelny et al., 2004; Anaya et al., 2006; Halkier and Gershenzon, 2006; Treutter, 2006; Cheng et al., 2007). Phytochemical defense in general will not be discussed further here; however, phytochemicals important for defense in the genus *Populus* will be described later (see section 1.6.1).

1.3 Perception of herbivory and initiation of signaling cascades

The induced expression of genes encoding products with defensive functions involves recognition of herbivore damage and subsequent defense signaling. Perception of feeding insects or mechanical damage is still poorly understood, but several wound- and herbivore-related cues have been discovered. Cell damage is predicted to release or generate cues that trigger the defense response, such as oligogalacturonic acids (OGAs), the polypeptide hormone systemin, and ATP. In addition, herbivore-derived chemical elicitors have been identified in insect saliva and regurgitant, including the enzymes β-glucosidase and glucose oxidase, the peptide inceptin and fatty acid-amino acid conjugates. The following section discusses recent advances in plant perception of herbivory. This results in initiation of a signaling cascade that activates the induced defense response in wounded tissues, as well as the generation of a systemic signal that

induces defense responses in systemic tissues. Downstream defense signaling will be discussed in section 1.4.

1.3.1 Plant perception of tissue damage (i.e. wounding)

It is not clear how plants perceive tissue damage, but several possible mechanisms have been suggested. OGAs are carbohydrate molecules that may be generated at insect feeding sites by hydrolysis of pectin from plant cell walls by plant or insect polygalacturonases (Bergey et al., 1999; Gatehouse, 2002). OGAs activate early components of defense signal transduction, including membrane depolarization, Ca^{2+} influx, and activation of a MAP kinase cascade (Gatehouse, 2002). In tomato, herbivore feeding and wounding also release systemin, an 18-amino-acid polypeptide (Ryan, 2000). Prosystemin, the precursor of systemin, may be liberated for proteolytic cleavage upon wounding or insect feeding, since it is compartmentalized within vascular phloem parenchyma cells (Narvaez-Vasquez and Ryan, 2004). Systemin initiates the defense response at exceptionally low (femtomolar) concentrations by binding to SR160, a plasma membrane receptor (Pearce et al., 1991; Scheer and Ryan, 1999, 2002). Although systemin defense signaling appears restricted to the Solaneae subtribe of the Solanaceae family, a search for defense signals in tobacco leaves identified hydroxyproline-rich glycopeptides with similar elicitor properties as systemin (Pearce et al., 2001; Rocha-Granados et al., 2005; Ren and Lu, 2006). These putative peptide hormones may be more prevalent in plants than the original tomato systemin. In addition to their potential roles as elicitors of wounding and herbivore feeding responses, OGAs and systemin also play roles further downstream in defense signaling (see section 1.4).

Extracellular ATP (eATP) has recently been proposed as another plant elicitor for wounding and herbivore feeding. In animals, eATP can function as a neurotransmitter, but only recently has ATP has been considered as a potential signal in plants (Demidchik et al., 2003). Upon wounding, ruptured plant cells release ATP into the extracellular matrix, which appears to initiate defense signaling by binding to a plant ATP receptor (Song et al., 2006). Exogenous ATP activates the early components of defense signal transduction and the threshold of eATP required to initiate signaling is lower than that measured at wound sites (Jeter et al., 2004; Song et al., 2006). Thus, ATP may be an

important plant signal molecule that allows plants to perceive wounding and insect herbivory.

1.3.2 Plant perception of insect-derived elicitor molecules

During feeding, insects deposit oral secretions on wound sites, and elicitors found in these secretions can trigger plant defense responses. Insect oral secretions consist of both saliva and regurgitant, from which several potent elicitors of plant defense responses have been identified. Such elicitors include fatty acid-amino acid conjugates (FACs), the enzymes glucose oxidase and β-glucosidase, and the peptide inceptin.

For most lepidopterans, the primary elicitors of plant defense in regurgitant are FACs. FACs are plant-derived C18 fatty acids that are conjugated to insect-derived glutamine or glutamic acid within insect alimentary tissues (Pare et al., 1998; Lait et al., 2003; Yoshinaga et al., 2005). FACs of different types are now known to be widespread among lepidopterans (Pohnert et al., 1999; Mori et al., 2003). FACs are not mobile defense signals but rather are restricted to feeding sites, where they bind a membrane-bound receptor (Truitt and Pare, 2004; Truitt et al., 2004). When applied to plant wound sites, FACs elicit various defense reactions, including changes in gene expression and volatile release (Alborn et al., 1997; Halitschke et al., 2001; Alborn et al., 2003; Halitschke et al., 2003; Roda et al., 2004). However, in some plant-pest interactions, FACs do not completely replicate responses induced by regurgitant, which suggests that regurgitant contains other elicitors capable of inducing defense responses.

A new peptide elicitor, termed inceptin, was recently identified from oral secretion of *Spodoptera frugiperda*. Inceptin is a potent activator of defense responses in cowpea plants, and induces volatile emissions, phenylpropanoids and PIs (Schmelz et al., 2006). Inceptin appears to be generated as a proteolytic fragment of plant chloroplastic ATP synthase γ-subunit (cATPC) by insect-specific proteases. An additional three cATP fragments that are inceptin-related peptide variants have recently been identified and have similar elicitor activity as inceptin (Schmelz et al., 2007). Although evidence indicates that inceptins are processed by insect proteases in the gut, it is possible that small quantities are released by leaf damage and are concentrated in insect oral secretions. Overall, it is likely that regurgitant elicitors such as inceptins, FACs, and possibly as yet

undiscovered elicitors combine to determine the defense response that is elicited by insect regurgitant.

Insect saliva also affects plant defense responses, though unlike elicitors of insect regurgitant, glucose oxidase (GOX) present in insect saliva suppresses defense responses resulting in increased insect growth and survival (Musser et al., 2002; Musser et al., 2005). Salivary GOX reduces nicotine accumulation and volatile emission in tobacco, and PI accumulation in tomato (Musser et al., 2002; Musser et al., 2005; Delphia et al., 2006). GOX is deposited at feeding sites and oxidizes glucose to produce gluconic acid and H_2O_2, resulting in increased levels of H_2O_2 that are primarily responsible for suppression of defenses (Eichenseer et al., 1999; Musser et al., 2002; Peiffer and Felton, 2005). Thus, insect elicitors are capable of both inducing and suppressing components of plant defense responses.

Although numerous potential elicitors of insect origin have been reported, how they relate to the wound response is poorly understood. Some studies suggest that plants respond differently to wounding compared with herbivory (Walling, 2000; Gatehouse, 2002; Tumlinson and Lait, 2005); however, it is not clear how the insect-derived elicitors modulate defense signaling to generate insect-specific responses. Interestingly, a recent study in lima bean using a "mechanical caterpillar" engineered to closely mimic insect feeding damage over an extended period found that both the spatial and temporal extent of physical damage affects the composition of released volatiles (Mithofer et al., 2005). There is thus still much that needs to be resolved in terms of how plants respond to herbivore feeding.

1.4 Wound signal transduction and systemic signaling

Plant intracellular signaling is initiated within minutes of perception of wounding or insect feeding and includes an influx of Ca^{2+} (Chico et al., 2002), plasma membrane depolarization (Felix and Boller, 1995; Schaller and Oecking, 1999), and activation of a MAP kinase cascade (Stratmann and Ryan, 1997; Wu et al., 2007). These early signaling events generate secondary messenger signal molecules, which induce a defense response in neighboring plant cells (Gatehouse, 2002). Plant polygalacturonases are induced by early defense signaling and release OGAs from cell walls, which in turn activate an H_2O_2

burst (Bergey et al., 1999; Orozco-Cardenas et al., 2001). The H_2O_2 diffuses to surrounding tissues and triggers expression of genes whose products increase resistance to feeding insects. Early signals also activate jasmonic acid (JA) biosynthesis, a plant hormone required for local and long-distance defense signaling. The necessity of JA for successful activation of plant defense is exemplified by the numerous mutants of JA biosynthesis and perception that are deficient in many defense responses (Berger, 2002).

The initial JA increase initiates a rapid JA amplification via positive feedback involving systemin, which increases JA to levels required for systemic signaling (Ryan and Moura, 2002; Wasternack et al., 2006). JA accumulates preferentially in vascular bundles, to which JA biosynthetic enzymes are localized (Hause et al., 2000; Hause et al., 2003; Stenzel et al., 2003). Accumulation of JA in vascular bundles suggests that JA, or a JA derivative, is the systemic defense signal, since the signal is transported via the phloem. Studies from several plants have shown that systemic signal movement is governed by vasculature and source-sink relations, which are determined by phloem architecture (Davis et al., 1991b; Orians et al., 2000; Schittko and Baldwin, 2003). The most convincing evidence for JA as a systemic defense signal was recently presented by Howe and co-workers, who used grafting experiments to elucidate the nature of the signal (Li et al., 2002; Lee and Howe, 2003; Li et al., 2005). Reciprocal grafts of mutants deficient in JA synthesis or JA perception demonstrated that the systemic signal is dependent on JA synthesis in wounded tissues and JA perception in systemic unwounded tissues, suggesting that JA, or a JA derivative, may be the transmissible wound signal (Li et al., 2002; Li et al., 2005).

1.5 Expression profiling as a tool to study plant defense responses

Transcript profiling, which examines transcript levels for large numbers of genes simultaneously, has developed into a useful tool for studying plant stress responses like insect herbivory. Many studies have shown that transcript profiling is an effective tool for comparing plant responses elicited by different treatments simulating herbivory as well as the responses induced in different tissues. Such studies of plant defense have compared defense responses elicited by mechanical damage, insect herbivory and caterpillar regurgitant (Reymond et al., 2000; Halitschke et al., 2003; Reymond et al., 2004),

responses induced in damaged and systemic tissues (Strassner et al., 2002; Reymond et al., 2004), and responses induced by specialist and generalist herbivores, as well as insects of different feeding guilds (Heidel and Baldwin, 2004; Reymond et al., 2004; De Vos et al., 2005). For example, early studies in *Arabidopsis* detected few differences in transcript profiles between wounding and insect derived-elicitors or feeding (Reymond et al., 2000; Reymond et al., 2004). Halitschke et al. (2003) used DNA microarrays to show that FAC elicitors present in *Manduca sexta* regurgitant are responsible for most of the insect-specific gene induction of regurgitant in *Nicotiana attenuata*. Comparison of transcript abundance in local and systemic tissues of wounded tomato revealed attenuated systemic expression of JA biosynthetic genes but comparable expression of genes encoding defense proteins, such as PIs and PPO, in wounded and systemic tissues (Strassner et al., 2002). However, in *Arabidopsis* and *Nicotiana attenuata* challenged by *Pieris rapae* and *Manduca sexta* feeding, respectively, systemic and local responses were similar (Heidel and Baldwin, 2004; Reymond et al., 2004). Other microarray studies showed that plant responses to specialist and generalist caterpillar folivores have similar expression profiles (Heidel and Baldwin, 2004; Reymond et al., 2004). By contrast, phloem-feeding aphids and cell-content feeding mirid bugs trigger markedly different plant defense responses compared to leaf-chewing herbivores (Heidel and Baldwin, 2004; De Vos et al., 2005). These studies show the value of transcript profiling, and demonstrate that similarities and differences can be detected among responses to different treatments, which provide valuable insights about plant perception of herbivory.

Transcript profiling studies have also generated a catalog of hundreds of putative defense genes that are induced in response to wounding or herbivory. Many hundreds of genes are induced by wounding and insect damage in *Arabidopsis* (Cheong et al., 2002; Reymond et al., 2004), at least 500 tobacco genes are estimated to be affected by herbivory (Halitschke et al., 2001), and more than a thousand genes from hybrid poplar are induced after abrasion damage (Smith et al., 2004) or herbivory (Ralph et al., 2006a). These inducible genes are candidates for future studies to determine how the gene products confer insect resistance. For example, in *Nicotiana attenuata*, threonine deaminase (TD) was first identified as strongly induced by herbivory from differential display screens (Hermsmeier et al., 2001) and has recently been shown to be an

antinutritive defense enzyme in tobacco and tomato (see section 1.2.3) (Kang et al., 2006; Chen et al., 2007).

1.6 Inducible defense in *Populus*

Populus species are often subject to dramatic defoliations by insect pests, and like other plants, they have evolved active defense responses. The inducible defenses of *Populus* have been extensively studied at the molecular and ecological levels. Studies by Lindroth and co-workers have examined the interaction of several insect herbivores with *P. tremuloides*, and shown the role of poplar phytochemicals for anti-herbivore defense (see below). Furthermore, the induced defenses of poplar have been directly shown to increases pest resistance. Poplar saplings previously subjected to herbivory, wounding, or caterpillar regurgitant are poorer hosts for gypsy moth larvae (*Lymantria dispar*) and sustain up to 71% less damage in subsequent attacks (Havill and Raffa, 1999). Herbivory was also shown to increase resistance to subsequent pest attacks by FTC and whitemarked tussock moth (*Orgyia leucostigma*) (Robison and Raffa, 1997; Glynn et al., 2003). The basis of pest resistance in poplar will be discussed in this section, with specific references to poplar defense phytochemicals and proteins.

1.6.1 Phytochemical defense

Poplar defenses include phytochemicals, which primarily consist of a variety of phenolic compounds, and have been extensively studied from both chemical and ecological perspectives (Lindroth and Hwang, 1996). The *Populus* genus is distinguished by a diversity of salicylate-based phenolic glycosides, which can comprise as much as 7% of foliage dry weight in *P. tremuloides* (Lindroth and Hwang, 1996). The phenolic glycosides in *P. tremuloides* include salicin, which can be further esterified (salicortin), benzoylated (tremuloidin), or both (tremulacin) (Lindroth and Hwang, 1996). Other related glycosides are present at varying levels in other species (Palo, 1984). The structural features of phenolic glycosides alter biological activity, as the esterified derivatives salicortin and tremulacin have the greatest activity (Lindroth et al., 1988). Overall, high levels of phenolic glycosides correlate with reduced larval growth and development, and are potent toxins for FTC, gypsy moth, tiger swallowtail (*Papilio*

glauca) and large aspen tortrix larvae (*Choristoneura conflictana*) (Lindroth and Hwang, 1996; Constabel and Major, 2005). Although phenolic glycosides cause lesions in insect midguts (Lindroth et al., 1988), the biologically active molecule is not definitively known because they may degrade during feeding. For example, salicortin is entirely degraded to salicin and catechol in the alkaline gut of lepidopteran larvae (Ruuhola et al., 2001; Constabel and Major, 2005). Catechol is an excellent substrate for PPO and thus wound-inducible PPO could enhance the toxic effects of phenolic glycoside degradation products (Haruta et al., 2001b). There is little evidence that the phenolic glycosides are induced by wounding; however, genotype, season and environment have a profound effect on the levels of phenolics and may confound studies of their inducibility (Lindroth and Hwang, 1996; Constabel and Major, 2005).

The second major class of defensive phytochemicals found in *Populus* is the proanthocyanidins, or condensed tannins, which are polymers of flavonoids. These can accumulate to as high as 18% of foliage dry weight in *P. tremuloides* but vary widely depending on species (Lindroth and Hwang, 1996). Condensed tannins have been shown to be negatively correlated with insect performance, although their impact is modest (Hemming and Lindroth, 1995; Hwang and Lindroth, 1997; Donaldson and Lindroth, 2004). The mechanism of condensed tannin toxicity has not been established; it has been suggested that biological activity may be due to oxidative activation (Lindroth and Hwang, 1996), but recent evidence suggests that condensed tannins are potential antioxidants in insect guts (Barbehenn et al., 2006). The effectiveness of condensed tannins as a defense depends on chemical structure variations, plant source, and insect gut environment (Ayres et al., 1997; Constabel and Major, 2005). Condensed tannin synthesis is induced by wounding and herbivory in *P. tremuloides* (Osier and Lindroth, 2001; Peters and Constabel, 2002), and expression analysis of genes encoding flavonoid biosynthesis enzymes has shown that the pathway leading to condensed tannin synthesis is inducible (Peters and Constabel, 2002; Tsai et al., 2006). As with phenolic glycosides, there is extensive variability of condensed tannin levels due to genotype, season and environment (Constabel and Major, 2005).

1.6.2 Molecular analysis of inducible defense

Wound-induced gene expression in poplar was first described by Gordon and co-workers, who isolated genes encoding an inducible Kunitz trypsin inhibitor (KTI), several chitinases, and a vegetative storage protein (Parsons et al., 1989; Bradshaw et al., 1990; Davis et al., 1991a; Davis et al., 1993). Subsequent work in this laboratory has identified PPO as an inducible defense protein of poplar (Constabel et al., 2000; Haruta et al., 2001b). Additional and more detailed studies have subsequently characterized inducible poplar defenses at the transcript, protein, and enzyme activity levels. Recent transcript profiling studies of poplar herbivore and wound responses have generated even larger lists of induced genes with possible roles in defense, but these have yet to be characterized (Smith et al., 2004; Lawrence et al., 2006; Ralph et al., 2006a). Those wound-inducible poplar genes that have been shown to encode products that directly impact insect herbivore performance are described below.

The only family of poplar PIs that has been identified as wound-inducible to date is the KTI family. In hybrid poplar, wounding induces expression of *win3*, the KTI first identified by Bradshaw et al. (1990), in both systemic and damaged leaves (Bradshaw et al., 1990; Davis et al., 1991b; Hollick and Gordon, 1993). Subsequent expression of WIN3 in tobacco leaves demonstrated a modest but significant reduction in growth of *Heliothis virescens*, consistent with a role for WIN3 in anti-insect defense (see section 1.2.1) (Lawrence and Novak, 2001). In *P. tremuloides*, Haruta et al. (2001a) cloned two KTIs (*TI1* and *TI2*) orthologous to *win3*, and showed wound-induced expression for *TI2* in damaged and systemic leaves. Wounding also induced TI2 protein accumulation to levels consistent with those of induced PI proteins in other plants (Jongsma and Bolter, 1997; Haruta et al., 2001a). Therefore, *win3* in hybrid poplar and *TI1/2* in aspen appear to have similar anti-insect functions in these two *Populus* species.

Analyses of the *win3* and *TI1/2* loci suggest that they are hypervariable and are members of a large, rapidly evolving gene family (Hollick and Gordon, 1993; Haruta et al., 2001a). These results have been confirmed by recent molecular evolutionary studies, which found evidence of gene duplications and positive selection pressures (Ingvarsson, 2005; Talyzina and Ingvarsson, 2006). Recent work in our laboratory has identified three

additional wound-induced KTIs from this family in hybrid poplar (Christopher et al., 2004), which consists of 20-30 genes based on estimates from the completed genome sequence (Tuskan et al., 2006). This large family of rapidly evolving KTIs, which are likely to reduce performance of poplar pests, could indicate an evolutionary arms race with insect herbivores.

In hybrid poplar and *P. tremuloides*, wounding or herbivory increase PPO activity, and studies of PPO gene expression from both species suggest a defensive function for poplar PPO (Constabel et al., 2000; Haruta et al., 2001b). Biochemical analysis has shown that PPO is expressed as a latent enzyme, but is activated by passage through the FTC gut (Wang and Constabel, 2004a). Poplar PPO is stable and resists proteolysis in FTC, consistent with anti-insect defense. Poplar contains as many as 10-12 PPO genes, and work in this laboratory to characterize expression of three of these PPOs showed that two are wound-inducible, but they are expressed in different plant tissues (Wang and Constabel, 2004b). Moreover, the two wound-inducible PPOs have distinct biochemical properties, including pH optimum and substrate specificity (Wang and Constabel, 2003). To directly test if poplar PPO negatively affects lepidopteran pests, one of the inducible PPOs was overexpressed in hybrid aspen (*P. tremula* × *P.alba*). FTC larvae feeding on PPO-overexpressing leaves had reduced growth and increased mortality, although these effects were only observed for less vigorous larvae hatched from older egg masses (Wang and Constabel, 2004a). These experiments provide direct proof of the anti-herbivore defense of poplar PPO (see section 1.2.2).

Early screens of wound-induced hybrid poplar cDNA libraries identified two distinct poplar chitinases, *win6* and *win8*, with about 50% amino acid sequence identity and which are strongly wound-inducible in damaged and systemic leaves (Parsons et al., 1989). Additional analyses showed that these genes belong to multigene families (Parsons et al., 1989; Davis et al., 1991a). Chitinase activity was subsequently shown to increase in poplar leaves following wounding, and transgenic tobacco expressing WIN6 have enhanced chitinase activity (Clarke et al., 1998). It was initially suggested that these wound-induced chitinases could provide protection against opportunistic fungal pathogens. However, a recent study showed that WIN6 decreased growth and development of *Leptinotarsa decemlineata* fed tomato plants expressing the poplar WIN6

protein (Lawrence and Novak, 2006). This provides direct evidence for anti-herbivore defense of poplar chitinases and suggests that these enzymes attack the PM of insect pests (see section 1.2.6).

1.6.3 Populus as a model species for molecular analysis of defense

Populus has become a major focus of plant genomics research, and a model species for answering questions that cannot be easily addressed in herbaceous plants, including *Arabidopsis* and rice, the other major model systems for plant biology and genomics. Important insights are not only evolving from studies of tree-specific traits such as wood formation, perennial growth and seasonality, but also questions such as flowering control and biotic and abiotic interactions. Genomics resources have developed rapidly for *Populus* in the last decade. The *P. trichocarpa* genome is fully sequenced (Tuskan et al., 2006) and large expressed sequence tag (EST) collections are publicly available (Jansson and Douglas, 2007). Poplar cDNA arrays are presently being used to investigate a variety of processes relevant to woody plants and perennials, including autumn senescence and wood development (Andersson et al., 2004; Schrader et al., 2004; Moreau et al., 2005), as well as abiotic and biotic stress resistance (Gu et al., 2004; Smith et al., 2004; Ralph et al., 2006a). Much of this expression profiling data for *Populus* is now publicly accessible (Sjodin et al., 2006). Finally, the large number of naturally-occurring poplar insect herbivores make *Populus* a powerful system in which to study plant-herbivore interactions at the molecular and genomic levels.

1.7 Objectives and Rationale

When this study began, our knowledge of inducible defense in poplar was limited. The goals of this research were thus to further characterize the herbivore defense response of hybrid poplar at the molecular level, with the aim of better understanding the biochemical mechanism of defense. Previous work in this lab identified a set of defense-related genes from poplar with an EST sequencing project of leaves undergoing a defense response. This study began by building upon this EST resource with the construction of macroarrays to be used for transcript profiling of poplar inducible defense. Specifically, the objectives of this research were (1) to use transcript profiling with macroarrays to

study herbivore-induced changes in gene expression of hybrid poplar; (2) to compare the poplar defense response induced by mechanical wounding vs. FTC regurgitant, as well as local vs. systemic defense responses (Chapter 2 and 3); (3) to biochemically characterize one type of defense protein, the Kunitz trypsin inhibitor family of poplar (Chapter 4), whose members likely have anti-insect properties and are among the most strongly induced genes in the poplar defense response; and (4) to examine the inhibitor activity of the KTIs against FTC midgut proteases, and the effects of TI3, the strongest poplar inhibitor, on insect digestive physiology and consequent growth and development (Chapter 5).

2 Molecular analysis of poplar defense against herbivory. Comparison of wound- and insect elicitor-induced gene expression

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2.1 Introduction

Under selective pressure by herbivores, plants have evolved a variety of physical and biochemical defense mechanisms. A common defense strategy is the active deployment of inducible defenses, which can include both proteins and secondary metabolites whose synthesis is triggered by herbivore damage (Walling, 2000; Baldwin et al., 2001; Gatehouse, 2002; Kessler and Baldwin, 2002). Inducible defenses often act as toxins, antifeedants, or antinutrients; for example, many plants synthesize herbivore-induced proteinase inhibitor proteins which inhibit insect digestive enzymes and have directly toxic effects (Ryan, 1990). Other antinutritive defenses include the oxidative enzymes polyphenol oxidase (PPO), peroxidase and lipoxygenase, thought to act by destroying or modifying essential amino acids and fatty acids (Duffey and Felton, 1991). In some species, the induced defense arsenal includes secondary metabolites such as alkaloids, terpenoids, or phenolics (reviewed in Walling, 2000; Kessler and Baldwin, 2002). Herbivory also stimulates the synthesis and release of terpenoid, aromatic and aliphatic volatiles, potential signals which can attract predators and parasitoids of the herbivore and thus contribute to defense (Arimura et al., 2005). It is significant that herbivore defenses are often induced beyond the site of insect feeding, in undamaged leaves of damaged plants. These leaves thus gain considerable resistance to herbivory despite not being directly damaged (Havill and Raffa, 1999). This systemic defense response and its regulation has been especially well characterized in tomato, where it is now known that jasmonic acid (JA) has a prominent role in systemic signaling (Bergey et al., 1996; Ryan, 2000; Howe, 2004; Schilmiller and Howe, 2005).

A number of insect-derived elicitors that are recognized by plant cells and trigger defense reactions have been discovered in insect regurgitants. They include both proteins, such as β-glucosidase, or fatty acid-amino acid conjugates (FACs)(Tumlinson and Lait, 2005). The first FAC with elicitor activity to be identified was *N*-hydroxylinolenoyl-L-

glutamine and named volicitin based on its ability to trigger volatile release (Alborn et al., 1997). Other potent FAC elicitors are *N*-linolenoyl-L-glutamine and *N*-linolenoyl-L-glutamic acid (Tumlinson and Lait, 2005). FACs have since been shown to be widespread among all caterpillar species examined to date, including species of the families Noctuidae (e.g. *Spodoptera* spp.), Geometridae and Sphingidae (e.g. *Manduca* spp.)(Pohnert et al., 1999; Halitschke et al., 2001; Mori et al., 2001; Mori et al., 2003). They induce various defense reactions, including changes in gene expression and volatile release (Alborn et al., 1997; Halitschke et al., 2001; Alborn et al., 2003; Halitschke et al., 2003; Roda et al., 2004).

Transcript profiling using DNA arrays is a powerful tool that is now being applied to investigate plant responses to insect feeding and wound signaling. DNA array studies have compared mechanical wounding and insect feeding, or damage by different insect feeding guilds (Heidel and Baldwin, 2004; Reymond et al., 2004; Voelckel and Baldwin, 2004). In *Arabidopsis,* arrays showed that the vast majority of herbivore-inducible transcripts are also upregulated by wounding (Reymond et al., 2000; Reymond et al., 2004). Similar experiments revealed that many wound-induced genes are induced by both osmotic stress and heat shock, or have led to the discovery of novel defense genes and biochemical pathways that respond to wounding and herbivory (Reymond et al., 2000; Cheong et al., 2002; Halitschke et al., 2003).

In hybrid poplar, wound-induced gene expression was first described by Gordon and co-workers, who isolated genes encoding an inducible Kunitz trypsin inhibitor (KTI), several chitinases, and a vegetative storage protein (Parsons et al., 1989; Davis et al., 1991a; Davis et al., 1993). We subsequently identified PPO as an inducible defense protein in this system (Constabel et al., 2000; Haruta et al., 2001b). Additional studies further characterized poplar induced defenses at the transcript, protein, and enzyme activity levels (Clarke et al., 1998; Constabel et al., 2000; Haruta et al., 2001a; Haruta et al., 2001b; Wang and Constabel, 2003). The efficacy of hybrid poplar induced defense has been directly demonstrated; poplar saplings previously subjected to herbivory, wounding, or caterpillar regurgitant are subsequently poorer hosts for gypsy moth larvae (Havill and Raffa, 1999). Moreover, the anti-herbivore effects of poplar PPO has been directly shown for forest tent caterpillar (FTC; *Malacosoma disstria*) using transgenic

poplar overexpressing this protein (Wang and Constabel, 2004a). Induced synthesis of poplar terpenoids, which may contribute to indirect defense, has also been described (Arimura et al., 2004). Furthermore, in *P. tremuloides,* herbivore- and wound-stress induces the expression of flavonoid biosynthesis genes, which is correlated with induced proanthocyanidin accumulation (Peters and Constabel, 2002).

Populus has become a major focus of plant genomics research. The *P. trichocarpa* genome is now fully sequenced (Brunner et al., 2004; Tuskan et al., 2004), and large expressed sequence tag (EST) collections are available. These provide a key resource that can be used for digital analysis of gene expression, gene discovery (Sterky et al., 2004), and to construct DNA microarrays. Poplar cDNA arrays are presently being used to investigate a variety of processes relevant to woody plants and perennials, including autumn senescence and wood development (Andersson et al., 2004; Schrader et al., 2004; Moreau et al., 2005), as well as abiotic and biotic stress resistance (Gu et al., 2004; Smith et al., 2004; Ralph et al., 2006a). The large number of naturally-occurring poplar insect herbivores makes *Populus* a powerful system in which to study plant-herbivore interactions at the molecular and genomic levels. Previously, we undertook a small-scale EST sequencing project in hybrid poplar (*Populus trichocarpa* × *P. deltoides*) to provide a set of defense-related genes and to obtain a broader view of the transcriptome in leaves undergoing a defense response (Christopher et al., 2004). A substantial number of ESTs were found to encode proteins involved in defense or secondary metabolism, and many were upregulated after wounding. Here, we report the use of macroarrays constructed from this EST set to examine the poplar leaf response to insect-derived cues from FTC. Our analysis revealed that for this collection of wound-induced genes, the responses to FTC-regurgitant and wounding are qualitatively similar, though quantitatively distinct.

2.2 Materials and Methods

Plant material

Poplar hybrid H11-11 (*Populus trichocarpa* × *P. deltoides*), originating from the University of Washington/Washington State University Poplar Research Program, were propagated from greenwood cuttings in Sunshine Mix #4 (Sungro, Seba Beach, AB,

Canada) in 0.25 L propagation containers (RootMaker, Huntsville, AL, USA). After plantlets had rooted and reached a height of approximately 10 cm, they were transplanted into 15 cm-diameter pots containing Sunshine Mix #4 plus slow-release nutrients (8.9 g L^{-1} controlled release 8-6-12 NPK plus micronutrients (Acer, Delta, BC, Canada), 0.458 g L^{-1} superphosphate 0–20–0 (Green Valley, Surrey, BC, Canada), 1.21 g L^{-1} Micromax Micronutrients (Scotts-Sierra, Marysville, OH, USA), and 4.75 g L^{-1} Dolomite lime (IMASCO, Surrey, BC, Canada)). Plants were maintained in the Bev Glover Greenhouse at the University of Victoria. All experiments were conducted between March and May. Supplemental lighting from 600 W high pressure sodium lamps was used to extend the photoperiod to 16/8 hr, and the temperature within the greenhouse was maintained at 25/18°C. Plants were watered daily with a solution containing 0.1 g L^{-1} 20-20-20 PlantProd fertilizer (Plant Products, Brampton, ON, Canada). All lateral shoots were pruned as they developed so that each plant consisted of a single main stem, no less than 2 weeks prior to wounding.

FTC-regurgitant collection

Regurgitant was collected from fourth- and fifth-instar FTC larvae found on aspen foliage (*P. tremuloides*) near Drayton Valley, AB, Canada. FTC-R was collected by micropipette, immediately frozen on dry ice, and stored at -80°C. The preparations were incubated at 100°C for 20 min to eliminate potential enzyme activity or enzymatic degradation of elicitors (Mattiacci et al., 1995; Mori et al., 2001; Alborn et al., 2003), and then filter-sterilized to eliminate microbial activity. FTC-R was analyzed by LC-MS for the presence of chemical elicitors by Dr. Amy Roda, Dr. Bernd Krock and Dr. Ian Baldwin (Max Planck Institute for Chemical Ecology, Jena, Germany).

Wounding and FTC-R treatments

Plants were 12 weeks old and 1 m tall with approximately 30 leaves when used for experiments. Leaves were mechanically wounded by crushing the margins of leaf blades, while for FTC-R treatments, FTC-R was applied to leaf punctures made with a fabric tracing wheel. A total of 100 µL of FTC-R was applied to 100 punctures (1 µL per puncture) over 10 rows per leaf. We first tested inducing activity of a range of FTC-R-

dilutions to establish an appropriate concentration for further experiments (Fig. 2-1). Leaves corresponding to LPI 10 (leaf plastochron index)(Larson and Isebrands, 1971) were treated with sterile ddH_2O or FTC-R diluted 1:1, 1:5, 1:20, 1:60, and 1:180 (v/v) with water, and harvested after 24 h for analysis. For subsequent experiments, FTC-R was diluted 1:5 (v/v) with sterile ddH_2O. FTC-R-treatment was compared between leaves treated with FTC-R and leaves treated with sterile ddH_2O (mock control). For macroarray experiments studying local responses, leaves of LPI 9-17 were treated three times, at 1 h-intervals, with either wounding or FTC-R. Leaves LPI 9-11 were harvested 24 h after start of the treatment, frozen in liquid nitrogen, and stored at -80°C until analyzed. For studying systemic responses, leaves of LPI 12-17 were treated 3 times (1 h-intervals), and untreated leaves of LPI 9-11 were harvested 24 h after start of the treatment. This design ensured that leaves designated as systemic were induced equally (Davis et al., 1991b), and were equivalent in age to directly-treated leaves.

RNA/protein extraction and hybridization

Total RNA was isolated from hybrid poplar leaves, quantified by UV absorbance, and quality verified on EtBr-stained agarose gels as previously described (Haruta et al., 2001a). RNA (10 μg per lane) was loaded onto 1.2% (w/v) agarose-formaldehyde gels, and blotted overnight onto Hybond-N^+ nylon membranes (Amersham Biosciences, Baie d'Urfé, PQ, Canada). RNA blots were probed with cDNA clones labeled with [α-^{32}P]dCTP (Rediprime II kit, Amersham). Hybridizations were performed at 65°C and were washed at high stringency according to Church and Gilbert (1984). The blots were detected with a Storm PhosphorImager (Amersham) and signal intensities were quantified using ImageQuant (Amersham). Ethidium bromide staining of RNA or blot hybridization with an actin cDNA probe was used to verify equal loading of lanes.

Macroarray analysis

For macroarray construction, 580 cDNA inserts were amplified by PCR. The majority of the cDNAs (569) were amplified from the unigene set of our EST library (Christopher et al., 2004), generated from systemic leaves of wounded hybrid poplar saplings (Constabel et al., 2000). The remaining 11 cDNAs were derived from a

suppression subtractive hybridization (SSH) library generated from systemic leaves of hybrid poplar saplings challenged by forest tent caterpillar (FTC; *Malacosoma disstria*)(J. Patton and C.P. Constabel, unpublished). A total of 10ng of DNA for each clone was spotted in duplicate onto pre-wet nylon membranes (Hybond N^+; Amersham) using a handheld multi-blot replicator as per the manufacturer's instructions (VP Scientific, San Diego, CA, USA). Care was taken to spot equal amounts of DNA so that we could gain additional information about transcript levels from our macroarray analysis. Membranes were incubated DNA side up on 0.2 N NaOH for 10 min, followed by 0.5 M Tris-HCl (pH 7.4)/0.5 M NaCl for 5 min, and cross-linked using a low energy UV cross-linker Stratagene, La Jolla, CA, USA). Membranes were rinsed in 2X SSPE/0.1% (w/v) SDS before air drying.

For array analysis, total RNA was isolated from three pooled leaves of each of three independent biological replicates (trees) for all four treatments (local wounding, systemic wounding, local FTC-R, systemic FTC-R) as well as the corresponding controls for each. Each replicate was analyzed on an individual macroarray. For synthesis of ^{33}P-labeled cDNA, poly(A)$^+$ RNA was isolated from 37.5 µg total RNA using Dynabeads Oligo $(dT)_{25}$ (Dynal Biotech, Lake Success, NY, USA), reverse transcribed using Superscript II (Invitrogen, Burlington, ON, Canada) and labeled with ^{33}P using a Rediprime II kit (Amersham). Macroarrays were hybridized at 65°C, washed at high stringency according to Church and Gilbert (1984), and exposed to PhosphoImager screens for 48 h. Images were scanned with a Storm PhosphoImager (Amersham), and the signals were quantified using ArrayVision 7.0 (Imaging Research, St. Catherines, ON, Canada). Background intensity surrounding each spot was calculated and subtracted from each spot. The average of duplicate spots was used for all downstream analyses. Signal values less than 1% of mean signal intensity were manually raised to avoid extreme expression ratios. Corrected spot intensities were normalized to the standard deviation of the entire array (Richmond and Somerville, 2000). Relative transcript abundance was calculated directly from intensities of the normalized signals. Test hybridizations comparing control plant mRNAs gave a correlation coefficient of 0.982. Normalized intensities from the three biological replicates were used to calculate average expression ratios.

A Student's *t*-test (paired, one-tailed) on $\log_2$-transformed data was used to determine statistical significance of expression ratios of each treatment and control pair. Q-values were calculated using R (http://www.r-project.org/; Storey and Tibshirani, 2003). Hierarchical clustering was performed with EPCLUST (http://ep.ebi.ac.uk/EP/EPCLUST/). Heat maps of *P*-values and transcript abundance were generated with Treeview (http://rana.lbl.gov/EisenSoftware.htm). χ^2 analyses were performed to assess significance of differences in the proportions of functional classes, as carried out in Smith et al. (2004).

Sequence analysis and data management were performed with Vector NTI Advance 9.0 (Invitrogen). To confirm the identity of genes, 20 of the most highly induced genes were confirmed by re-sequencing. To obtain full length sequences for candidate genes, the JGI poplar genome (http://genome.jgi-psf.org/Poptr1/Poptr1.home.html) was queried. Annotations of candidate genes were improved with similarity searches through BLASTP (Altschul et al., 1997) against the NCBI non-redundant protein database (http://www.ncbi.nlm.nih.gov/), the UniProt knowledgebase at EXPASY (http://www.expasy.org/), and the AGI protein database at TAIR (http://www.arabidopsis.org/). Conserved domains and motifs of unknown genes were identified through queries against the conserved domain database (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi). Predictions of nuclear localization signals were made with Prosite (http://www.expasy.org/prosite/) and PredictNLS (http://cubic.bioc.columbia.edu/predictNLS/).

2.3 Results

FTC-regurgitant induces poplar trypsin inhibitor gene expression

We previously demonstrated that mechanical wounding or feeding by forest tent caterpillar (FTC) triggers the hybrid poplar defense response. To establish if FTC-regurgitant (FTC-R) elicits a defense response in poplar leaves and if it could be used as an insect-derived cue for expression profiling, we collected regurgitant from FTC feeding on native *Populus* foliage. We applied a range of FTC-R dilutions to leaves using a tracing wheel, and measured transcript abundance of a Kunitz trypsin inhibitor (*PtdTI3*),

a robust marker of the poplar and aspen defense response (Haruta et al., 2001a; Christopher et al., 2004). These experiments showed that *PtdTI3* transcripts accumulated in a concentration-dependent manner in response to FTC-R, reaching maximum induction at a 1:5 dilution (Fig. 2-1A). This concentration was therefore chosen for all subsequent FTC-R-treatments.

The response of poplar leaves to FTC-R suggested that it must contain potent chemical elicitors that trigger defense responses in poplar cells. The regurgitant of other caterpillar species has been found to contain fatty acid-amino acid conjugates (FACs) which act as elicitors (Tumlinson and Lait, 2005). FTC-R was therefore tested for the presence of such FACs, together with regurgitant from *Manduca sexta* and *Spodoptera exigua* for comparison. LC-MS analysis revealed that volicitin (*N*-hydroxylinolenoyl-L-glutamine) was the major FAC in FTC-R, although minor peaks may represent additional FACs (Fig. 2-1B). Consistent with previous reports, volicitin was also detected in the regurgitant of *S. exigua* (Alborn et al., 1997; Pohnert et al., 1999), but only *N*-linolenoyl-L-glutamic acid was present in *M. sexta* (Halitschke et al., 2001; Alborn et al., 2003). Thus, these experiments revealed that volicitin is a component of FTC-R and could be a key elicitor of the poplar defense response during herbivory by FTC.

Expression profiling reveals that FTC-regurgitant elicits a strong defense response

To further study the transcriptional response of hybrid poplar leaves to FTC-R, we constructed macroarrays containing 580 cDNAs from an EST library, generated from induced hybrid poplar leaves (Constabel et al., 2000; Christopher et al., 2004). In addition to the FTC-R treatment, we also subjected leaves to mechanical damage using a tracing wheel or by crushing leaf margins with pliers (see below). We separately analyzed the response in treated leaves (local response) and untreated leaves on treated saplings (systemic response). Each control and induction treatment was analyzed using three independent biological replicates, and in addition each sample consisted of three pooled mature leaves. To be considered as differentially expressed between treated and control leaves, genes were required to meet two criteria: a $\geq$ two-fold change in expression (either up- or down-regulation), and a significance of P-value < 0.05 as determined by the Student's t-test for the three independent replicates. While a significant P-value alone has

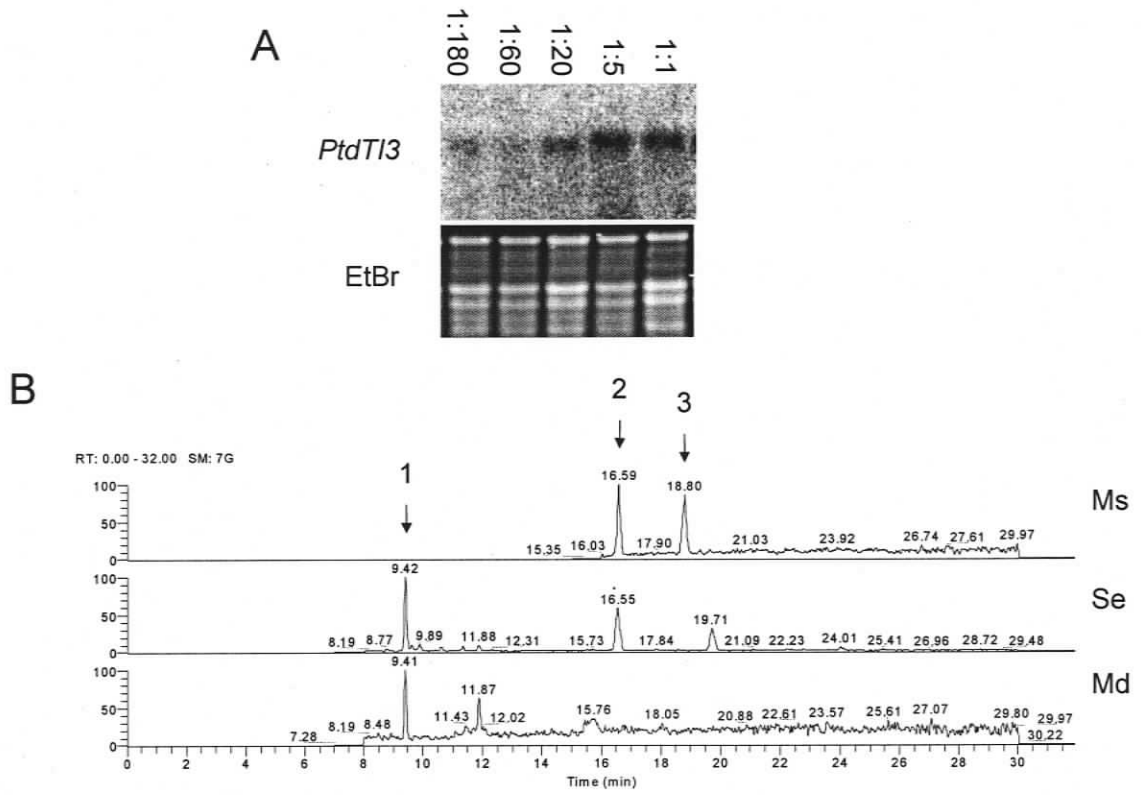

Figure 2-1. Analysis of FTC-R and FTC-R-induced expression of defense-related Kunitz trypsin inhibitor.

A. Accumulation of *PtdTI3* (Kunitz trypsin inhibitor 3) transcripts in poplar leaves in response to a gradient of FTC-R-dilutions. Dilutions of regurgitant were added to tracing wheel-punctures on leaves of LPI 10. Leaves were harvested at 24 h and analyzed by northern blot. EtBr, Ethidium bromide-stained gel used as a loading control.

B. LC-MS analysis of fatty acid-amino acid conjugates (FACs) from the regurgitant of *Manduca sexta* (Ms), *Spodoptera exigua* (Se), and *Malacosoma disstria* (Md). 1, *N*-hydroxylinolenoyl-L-glutamine (volicitin); 2, *N*-linolenoyl-L-glutamine; 3, *N*-linolenoyl-L-glutamate.

[LC-MS analysis of regurgitants carried out by Dr. Amy Roda, Dr. Bernd Krock, and Dr. Ian Baldwin at the Max Planck Institute for Chemical Ecology, Germany.]

often been used to determine differential expression, a two-fold expression threshold provides greater confidence that the expression ratios are meaningful. These relatively stringent criteria, however, may lead to conservative estimates of the extent of differential gene expression.

Compared to unwounded control leaves, FTC-R significantly upregulated 36 genes from our array. An additional 13 genes were induced by at least two-fold, but had *P*-values > 0.05. Some of the induced genes encoded previously identified wound-responsive genes. We therefore tested if the observed changes in gene expression could be due to the wounding caused by the tracing wheel during application of FTC-R (see Methods), and analyzed the gene expression ratios in tracing wheel/water-treated leaves relative to unwounded leaves. Only three genes were slightly but significantly upregulated in this comparison, confirming that the tracing wheel alone had only very minor effects on gene expression. Therefore, we were able to analyze the array data by calculating gene expression ratios of FTC-R treatment relative to the tracing wheel/water control treatment; this should provide an accurate representation of the effects of FTC-R alone and without the potential confounding effects of wounding. This analysis determined that 40 genes met our criteria for significant upregulation by FTC-R (Fig. 2-2A, see Supplemental Table 2-1). Thus our experiments clearly demonstrated that FTC-R induced a poplar defense response without significant mechanical wounding, presumably due to the volicitin and other chemical elicitors present.

One of our objectives was to compare the effects of FTC-R and mechanical wounding on gene expression. Since the tracing wheel caused almost no gene induction, we also performed a more severe wound treatment by crushing the leaf margins with pliers. This method was previously shown to cause a strong defense response in hybrid poplar leaves (Constabel et al., 2000; Christopher et al., 2004). Macroarray analysis of plier-wounded leaves determined that more than 100 genes were induced by this fairly severe wound stress (Fig. 2-2B, see Supplemental Table 2-1). This response was intense, as reflected in expression ratios of as high as 256-fold for defense genes such as chitinases and PPO. In both intensity and number of induced genes, the plier-wounding resulted in a stronger response than the FTC-R treatment (compare Fig. 2-2A and 2-2B). However, both treatments led to significant upregulation of many genes, and provided the

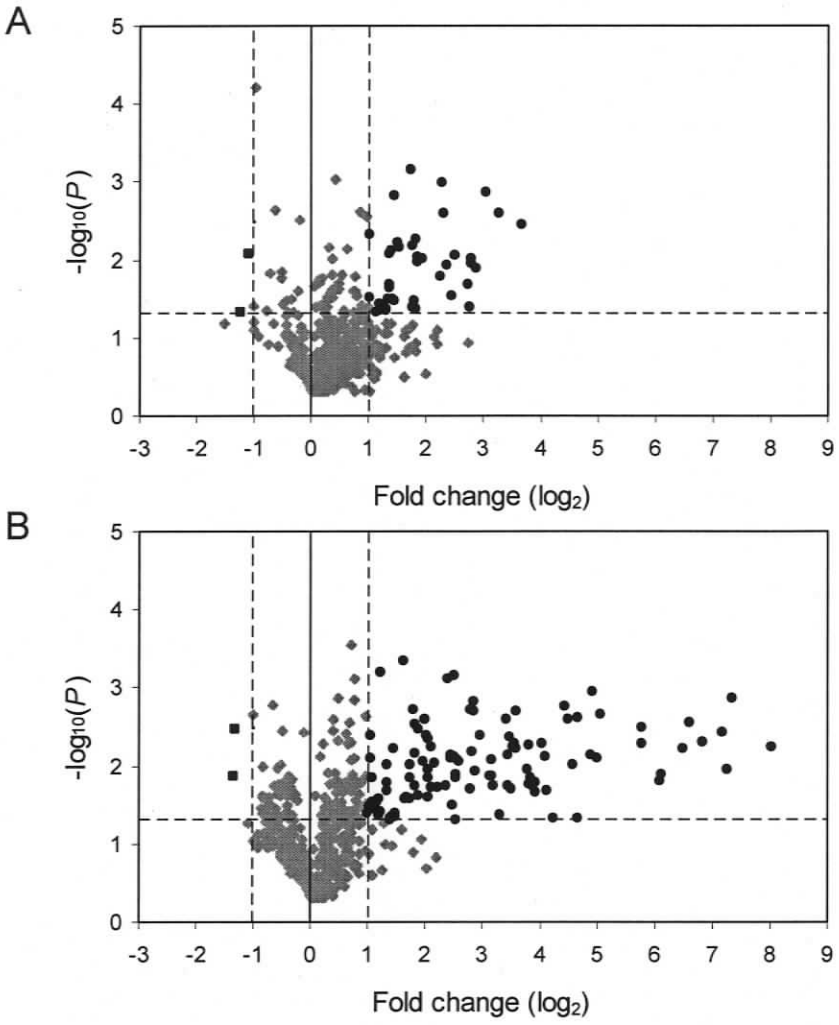

Figure 2-2. Summary of changes in gene expression in response to induction treatments as measured by macroarray analysis.

Volcano plots with gene expression ratios ($\log_2$ fold change) plotted against the negative $\log_{10}$-transformed *P*-values from a *t*-test calculation. (**A**) Gene expression ratios of FTC-R-treated to water-treated leaves, and (**B**) expression ratios of mechanical wounding to untreated leaves. Vertical dashed lines represent a two-fold change in gene expression threshold (induction or repression). Horizontal dashed line represents a significance level of $P = 0.05$. Black circles and squares represent genes with a statistically significant ($P < 0.05$) fold change of > 2 or < -2. All treatments were performed on leaves of LPI 9-17, and after 24 h leaves of LPI 9-11 were harvested. Experimental treatments and corresponding controls were harvested concurrently.

basis for a more in-depth comparison of these stresses (see below). Only two genes were found to be repressed by wounding or FTC-R (Fig. 2-2A and 2-2B top left quadrants). Other workers have reported a much larger number of genes that are down-regulated by simulated or actual herbivory (Cheong et al., 2002; Halitschke et al., 2003; Roda et al., 2004; Smith et al., 2004; Voelckel and Baldwin, 2004; Ralph et al., 2006a). However, Reymond et al. (2004) also reported only three of ~12,000 ESTs to be repressed after feeding by *Pieris rapae*.

Northern blots probed with a series of genes with distinct expression patterns validated our macroarray data; strongly induced (e.g. H1078), unaltered (e.g. H641), and mildly repressed (e.g. H272) expression patterns corresponded well between macroarray and northern data (Fig. 2-3A). Macroarray data were likewise validated across the treatments using *PtdTI3* as a probe (Fig. 2-3B). Further confirmation came from the replication of some genes on the macroarrays, which displayed a highly similar expression profile (data not shown). Because we standardized the quantity of DNA spotted on the macroarrays, we were also able to use the arrays to obtain a measure of total transcript abundance (transcript levels). Using this measure, macroarray- and northern-derived expression patterns were found to correlate well for *PtdTI3* and other genes (Fig. 2-3B). Thus we are confident our macroarrays provided a reliable measure of gene expression, and that they accurately captured the induction of genes by both mechanical wounding and FTC-R.

Comparative analysis of simulated herbivory treatments reveals that FTC-R induces many wound-induced genes

We compared our macroarray data from the four treatments (local wounding, systemic wounding, local FTC-R, systemic FTC-R) by cluster analysis for all genes whose expression was significantly regulated ($P < 0.05$, irrespective of fold change) in at least one treatment (Fig. 2-4); this set of genes encompassed approximately one-half of the ESTs spotted on our macroarrays. We generated color-coded "heat maps" for gene expression ratios (degree of induction, left panel), *t*-test significance (*P*-value, middle panel) and transcript abundance (right panel). The heat maps provide a graphical comparison of the expression data; differences in color intensity are observable between

Figure 2-3. Validation of macroarray data using northern blot analysis.
A. Accumulation of transcripts encoding selected genes, 24 h after wounding with pliers (W) or untreated control plants (C). Northerns were performed for three biological replicates with similar results; a representative northern is shown. Fold induction ± standard error is shown for macroarray data. Asterisks denote significance of induction calculated from a *t*-test (* - $P < 0.05$; ** - $P < 0.01$). Annotations of ESTs used as probes are: H272 (CN193029), carbonic anhydrase; H583 (CN193162), unknown gene, ZIM motif; H641 (CN193209), Rubisco; H892 (CN193384), unknown gene, DUF946 domain; H1078 (CN192556), endochitinase *win6.2C*; H1233 (CN192638), 11S cupin plant seed storage protein; H1441 (CN192724), apyrase; H1644 (CN192786), class 3 lipase. Actin is shown as a loading control. Numbers refer to EST identifiers, with GenBank accessions in parentheses.
B. Accumulation of *PtdTI3* (Kunitz trypsin inhibitor 3) mRNA in leaves of plants wounded with pliers (W) or treated with regurgitant (R). Leaves were harvested after 24 h from treated leaves (local) or untreated leaves on treated plants (systemic). Controls (C) were unwounded plants for wounding treatments, or plants mock treated with water for FTC-R-treatments. Top panel shows representative northerns from three biological replicates of all four treatments. Lower two panels show quantified transcript abundance from northerns and macroarrays, respectively. Error bars display standard error.

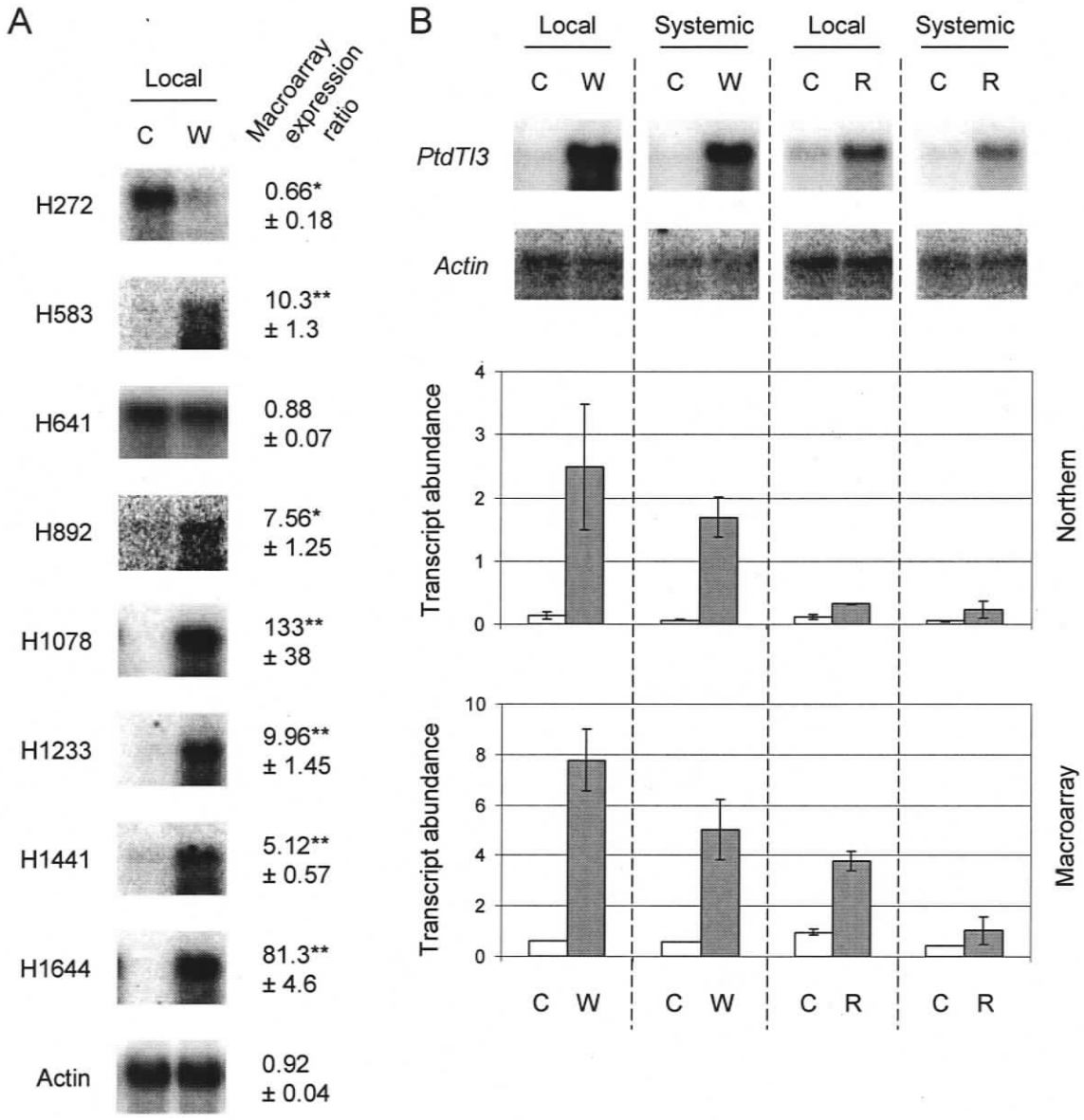
A
Local
C W
Macroarray expression ratio
H272
0.66* ± 0.18
H583
10.3** ± 1.3
H641
0.88 ± 0.07
H892
7.56* ± 1.25
H1078
133** ± 38
H1233
9.96** ± 1.45
H1441
5.12** ± 0.57
H1644
81.3** ± 4.6
Actin
0.92 ± 0.04
B
Local
Systemic
Local
Systemic
C W C W C R C R
PtdTI3
Actin
Transcript abundance
Northern
Transcript abundance
Macroarray
C W C W C R C R

Figure 2-3.

Figure 2-4. Heat map representing expression of all genes responding to wounding or FTC-R.

Hierarchical clustering was performed for all genes significantly regulated ($P < 0.05$) for at least one treatment. Heat maps illustrate fold-induction, *P*-values, and absolute expression levels. Genes clustered at the top are strongly induced (magenta) and putative defense genes, while those clustered at the bottom (green) are potential repressed genes. A discontinuous discretisation was introduced for the repressed genes to highlight the genes repressed twofold. Scale bars at bottom show color ranges: for fold induction (left panel), bright magenta shows a 100 fold increase of transcripts in treated leaves, black (delineated by a vertical line) shows no change of transcript level, and bright green shows a five-fold decrease of transcripts; for *P*-values (middle panel), dark pink shows $P < 0.001$, light pink shows $P = 0.05$, and blue shows $P = 0.5$; for transcript abundance (right panel), bright yellow shows high abundance and black shows low abundance. Black arrowheads denote known or suspected poplar defense genes, orange and magenta arrowheads denote highly induced genes annotated for primary metabolism and unknown function, respectively (see Tables 2-2 and 2-3), green arrowheads denote genes with predicted functions in photosynthesis, and blue arrowheads denote genes that were repressed twofold by one of the treatments. Poplar defense genes are labeled with brackets indicating the position of the genes on the heat map. *win6*, endochitinase *win6.2C*; *win8*, endochitinase *win8*; *PtdPPO1*, polyphenol oxidase 1; *VSP win4.5*, vegetative storage protein *win4.5*; *PtdTI3*, Kunitz trypsin inhibitor 3; *PtdTI4*, Kunitz trypsin inhibitor 4; *PtdTI5*, Kunitz trypsin inhibitor 5; *AOS*, allene oxide synthase; *PAL*, phenylalanine ammonia lyase; *13-LOX*, 13-lipoxygenase; *VSP pni288*, vegetative storage protein *pni288*.

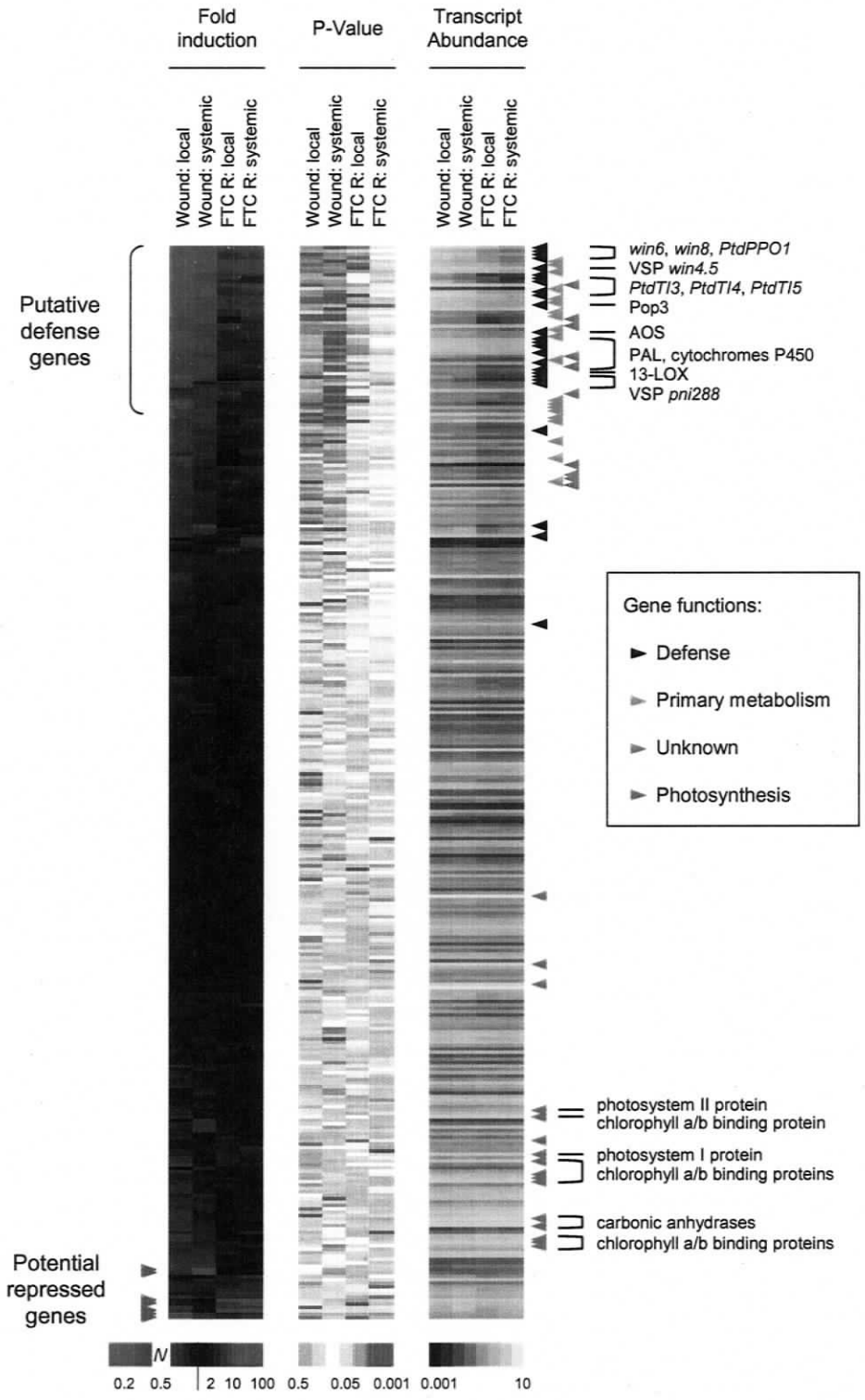
Fold induction
P-Value
Transcript Abundance
Wound: local
Wound: systemic
FTC R: local
FTC R: systemic
Putative defense genes
win6, win8, PtdPPO1
VSP win4.5
PtdTI3, PtdTI4, PtdTI5
Pop3
AOS
PAL, cytochromes P450
13-LOX
VSP pni288
Gene functions:
Defense
Primary metabolism
Unknown
Photosynthesis
photosystem II protein
chlorophyll a/b binding protein
photosystem I protein
chlorophyll a/b binding proteins
carbonic anhydrases
chlorophyll a/b binding proteins
Potential repressed genes
N
0.2 0.5 2 10 100
0.5 0.05 0.001
0.001 10

Figure 2-4.

the four treatments. All induced genes clustered together at the top of the panel, as seen by the most intense color pattern in this zone. Inspection of this set of genes revealed that plier wound-treatments showed more intense color, indicating stronger induction, greater significance, and higher transcript levels than FTC-R-treatments (compare color intensities of two leftmost lanes and two rightmost lanes in each panel). Thus the plier wound-treatments stimulated a stronger response than FTC-R-treatments, as already observed in Fig. 2-2 and 2-3. The group of genes at the top of the cluster contained the known poplar defense-related genes, including *win6.2C* and *win8* endochitinases (Davis et al., 1991a), *PtdPPO1* (Constabel et al., 2000), vegetative storage proteins (VSPs) *win4.5* and *pni288* (Davis et al., 1993; Lawrence et al., 2001), and trypsin inhibitors (TIs)(Christopher et al., 2004)(see below). Other genes found in this cluster included genes for jasmonate biosynthesis (allene oxide synthase, AOS; 13-lipoxygenase, 13-LOX) and genes likely involved in secondary metabolism (phenylalanine ammonia lyase, PAL; cytochromes P450)(Fig. 2-4); we previously identified some of these genes in the poplar wound-response (Christopher et al., 2004). In addition, the genes in this group were both the most strongly FTC-R- and wound-induced genes; thus there was an excellent correlation between both treatments for individual genes (see below).

Below the highly induced genes seen in Fig. 2-4, the majority of genes showed variable expression levels (right panel) and non-significant P-values (left and center panels), as well as apparent down-regulation. However, only seven genes showed significant repression in any of the treatments at $P < 0.05$. Among these were genes encoding peroxidase, catalase, ferredoxin, and a putative thiamine biosynthetic enzyme (Supplemental Table 2-1), but these need to be corroborated. Interestingly, genes with putative roles in photosynthesis were clustered at the bottom with the repressed genes, though none of these were significant (Fig. 2-4, arrows). The repression of photosynthetic genes as a component of the defense response has been reported by expression profiling studies (Halitschke et al., 2001; Hermsmeier et al., 2001; Heidel and Baldwin, 2004; Qu et al., 2004; Smith et al., 2004; Voelckel and Baldwin, 2004; Ralph et al., 2006a). This may be related to accumulation of jasmonic acid (JA) during the defense response, since JA has been shown to downregulate expression of photosynthetic genes (Creelman and Mullet, 1997).

The pattern of transcript abundance confirmed that both plier wounding and FTC-R had strong effects on the leaf transcriptome; the transcript levels of several wound-inducible genes were among the highest of any transcripts in the leaves (Fig. 2-4, right panel). For example, transcripts encoding chitinase *win6.2C*, VSP *win4.5*, *PtdTI3*, stress-related protein POP3/SP1-like, and several unknown proteins were among the 95th percentile in transcript abundance of all genes present on our macroarrays. This was comparable in strength to signals obtained for photosynthetic genes such as chlorophyll a/b binding proteins, Rubisco, carbonic anhydrase, Rubisco activase, and photosystem I subunits.

Poplar responses to severe wounding and FTC-R are similar

Previous studies in other species have suggested that some plant defense genes may be responsive to insect feeding but not to physical damage alone (Hermsmeier et al., 2001; Reymond et al., 2004), and that such responses can often be mimicked with insect-derived cues found in regurgitant (Schittko et al., 2001; Halitschke et al., 2003; Roda et al., 2004). Thus, we hypothesized that FTC-R may elicit an insect-specific defense response in poplar leaves. To further dissect differences between wound- and FTC-R-induced responses, we directly plotted the intersection of all genes that our criteria defined as induced by mechanical wounding or FTC-R using Venn diagrams. We found that the transcriptional response following FTC-R was a subset of the response induced by mechanical wounding, as this analysis showed that 38 of 40 (95%) FTC-R-inducible genes were also wound-inducible (Fig. 2-5A). The remaining two FTC-R-inducible genes (6%) were tentatively considered to be FTC-R-specific. In addition, the majority of wound-inducible genes (70 of 108, 65%) were not classified as induced by FTC-R and thus appeared to be wound-specific; however, this may reflect our threshold for defining induction (see below).

We examined the expression of those genes tentatively classified as wound- or FTC-R-specific further by hierarchical clustering (Fig. 2-5B). For this analysis, only expression data from local responses were used, and the generation of heat maps carried out as before. The top panels represent those genes classified as wound-specific while the bottom panels represent potential FTC-R-specific genes. Comparison of right and left

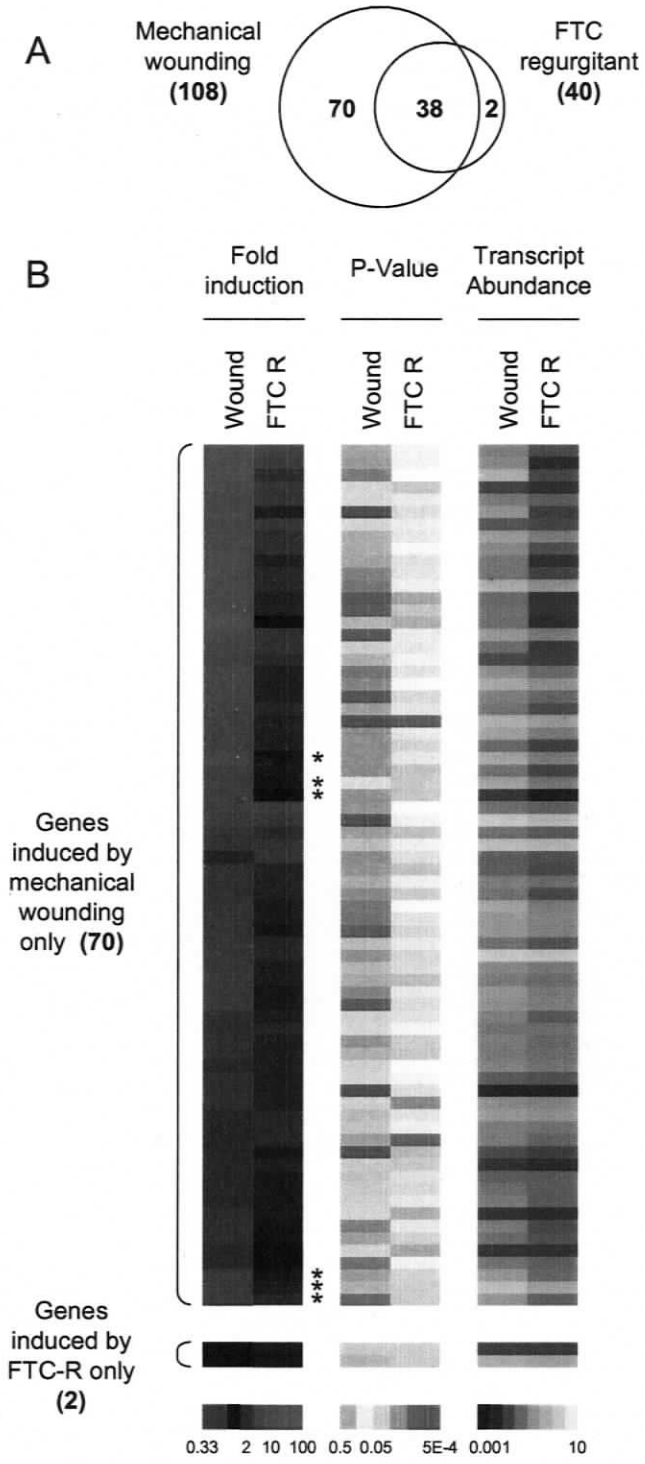

Figure 2-5. Comparison of gene expression after wounding or FTC-R treatment.

A. Venn diagram representing distribution of transcripts significantly induced by plier wounding or FTC-regurgitant on macroarrays; for clarity, only the analysis of gene expression in local leaves is shown.

B. Heat map of hierarchical clustering performed for genes classified as induced by wounding only or regurgitant only. Representation of average fold induction, *P*-values, and transcript abundance for local and systemic data are as described in Figure 2-4. Asterisks represent genes apparently preferentially induced by wounding.

lanes of each panel indicated that the majority of wound-specific genes also appeared to be induced by FTC-R but with lower or non-significant inductions (< 2-fold, or $P >$ 0.05); they thus did not meet the threshold for differential expression (Fig. 2-5B, top panels). Moreover, the pattern of transcript abundance of these genes as seen by the overall banding pattern was roughly similar in both treatments (Fig. 2-5B, top right panel). Two subgroups of genes appeared to exhibit strong wound-inducibility with no apparent response to FTC-R (Fig. 2-5B, asterisks). Subsequent northern analysis, however, showed that these genes exhibited very low levels of FTC-R-inducibility (data not shown). Thus, these genes should be considered to be preferentially expressed by wounding, perhaps with differences in induction kinetics that masked FTC-R-induced gene expression on the array. More detailed time course experiments will have to be carried out to test this.

Two genes, encoding mannose pyrophosphorylase and a Pop3-like protein, showed stronger induction by FTC-R than wounding, and are thus potential FTC-R-specific genes (Fig. 2-5B, bottom panels). Northern analysis again showed that wounding did in fact weakly induce these genes (data not shown). Thus our analysis failed to detect any exclusively wound-induced or FTC-R-induced genes, although several genes appeared to be preferentially induced by either stress. Overall, we conclude that the responses to FTC-R and wounding, as monitored by our suite of genes, differ quantitatively but are qualitatively similar.

Induced gene expression patterns in local and systemic leaves show extensive overlap

Upon insect challenge, hybrid poplar saplings induce defense genes such as PPO and chitinases systemically (Parsons et al., 1989; Constabel et al., 2000). Therefore we investigated induced gene expression patterns in systemically induced leaves. Much like the directly treated leaves, systemic leaves of wounded saplings showed a strong transcriptional response, and we identified 91 genes as significantly induced in these leaves. Fewer genes (22) met our criteria for induction in systemic leaves of FTC-R-treated saplings (see Supplemental Tables 2-1 and 2-2), and thus we did not include FTC-R treatments in this analysis. To compare the local and systemic wound-responses of poplar, we plotted the intersection of genes found to be induced in both local and

systemic leaves, and found an extensive overlap between local and systemic responses: 85 genes, comprising 79% of locally-inducible genes and 93% of systemically-inducible genes were induced in both types of leaves (Fig. 2-6A). Inspection by cluster analysis of the subsets of potential local-specific and systemic-specific genes emphasized the similarity of both responses (Fig. 2-6B). It also revealed that no genes were uniquely induced in local or systemic leaves, although some may be preferentially expressed in either group. Therefore, we conclude that for our set of genes there are no major qualitative differences in the defense response of local and systemic leaves.

The most highly FTC-R- and wound-induced genes are involved in defense, secondary metabolism and primary metabolism

A major objective of this study was the identification of novel induced defense genes from poplar leaves, as part of our long-term aim of a global characterization of poplar defense. Expression profiling studies in other plants have successfully identified many wound- or herbivore-induced genes and pathways, particularly during early events in the defense response. By studying the defense response at a 24 h time point, we aimed to focus on later events and to identify genes directly responsible for insect resistance.

A list of combined FTC-R- and wound-induced genes with the highest fold-induction demonstrated that known defense-related poplar genes, including endochitinases, PPO, TIs and the VSP *win4.5*, are the most strongly FTC-R-induced genes (Table 2-1). Genes encoding enzymes for octadecanoid synthesis, phenylpropanoid metabolism, or cytochrome P450 were also strongly induced and comprised functional categories which were well-represented in our EST set (Christopher et al., 2004). In addition, we identified a number of very responsive genes with putative roles in primary metabolism and not previously known to be involved in defense, although many were also identified by Ralph et al. (2006a) in their study of FTC-induced gene expression. These include genes encoding enzymes of carbohydrate, lipid, and phosphate metabolism, as well as genes of unknown function (Table 2-1). Comparison of the FTC-R- and wound-induced rankings revealed that they are generally similar. For example, the seven top genes are ranked within 1-10 for both FTC-R- and wound-inductions. This

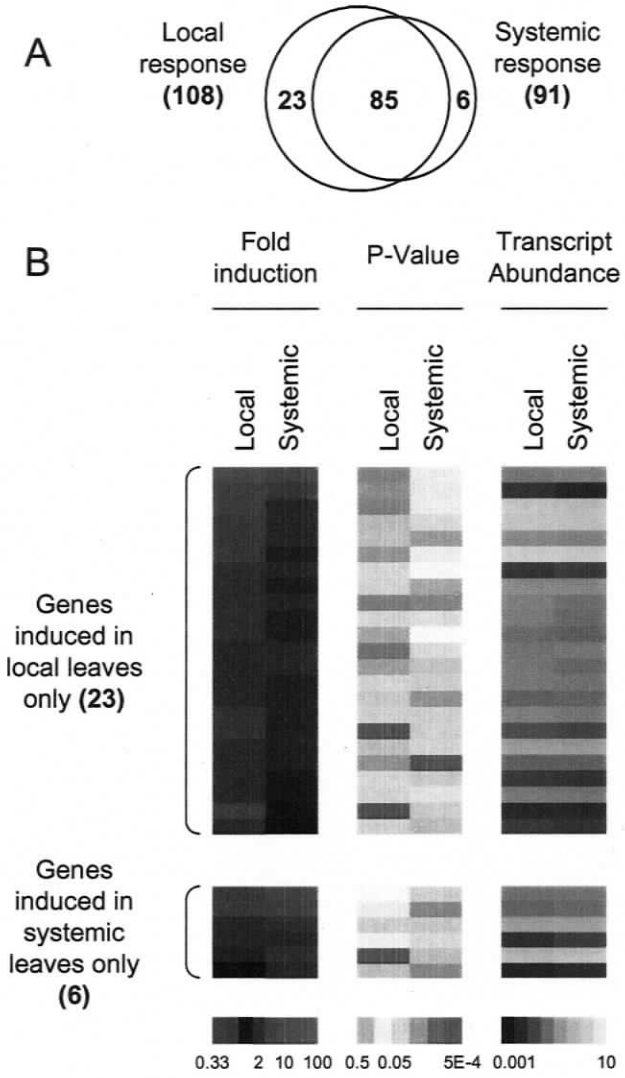

Figure 2-6. Comparison of local and systemic gene expression.

A. Venn diagram representing the distribution of significantly induced transcripts in treated (local) or untreated (systemic) leaves on macroarrays; for clarity, only the analysis of gene expression from mechanical wounding is shown.

B. Heat map of hierarchical clustering performed for genes classified as induced locally only or systemically only. Representation of average fold induction, *P*-values, and transcript abundance for wounding and FTC-regurgitant are as described in Figure 2-4.

supports our earlier conclusion that the FTC-R and wound-responses are qualitatively similar for this set of genes. A significant exception appeared to be the KTI genes (*PtdTI3* and *PtdTI4*) and the *pni288* VSP, which were both strongly induced by wounding but less so by FTC-R (Table 2-1). While FTC-R did induce *PtdTI4* and *pni288*, these inductions were not significant; if the *P*-values for *PtdTI4* and *pni288* are disregarded, their fold inductions by FTC-R rank as 20 and 29, respectively. These genes may thus be preferentially induced by wounding or their induction blocked by FTC-R. This will require more detailed experiments to be corroborated.

Further inspection of Table 2-1 indicated that genes classified into defense or secondary metabolism, including several cytochromes P450, were over-represented among genes induced by both simulated herbivory treatments. These differences in representation were significant, except for the FTC-R-response in systemic tissues (due to small sample size, n=22). While the macroarrays contained only ~4% each of defense or secondary metabolic genes, defense-related genes represented 9 - 15% of induced genes (χ^2 analysis, $P < 0.01$) and secondary metabolism represented 11 - 21% (χ^2 analysis, $P < 0.01$) of induced genes. Ralph et al. (2006a) also observed that secondary metabolism genes are activated in FTC-treated poplar leaves. Thus it appears that secondary compounds are likely important for poplar defense.

FTC-R and wounding strongly induced several genes annotated for roles in primary metabolism as well as genes of unknown function

Several of the most strongly wound- and FTC-R-inducible genes had predicted functions in primary metabolism, and others encoded proteins with no known functions. To confirm our annotation and gain additional information on possible functions, we searched the recently sequenced genome of *P. trichocarpa* (http://genome.jgi-psf.org/Poptr1/Poptr1.home.html) to obtain full-length sequences. Public databases (NCBI, UniProt, AGI) and databases for conserved domains (CDD, Interpro, ProSite) were then re-queried with these full-length genes in order to refine the earlier annotation and to ensure that no potential motifs or functions had been overlooked. As a functional group, primary metabolism encompassed 17.6% of the genes represented on the macroarrays, but up to 29% of the wound- and FTC-R-inducible gene set (not significant,

Table 2-1. Comparison of most strongly FTC-R- and wound-induced genes from macroarray analyses[a]

Putative function	GenBank accession	JGI gene model[b]	AGI accession[c]	E-value[d]	FTC-R rank[e]	Wound rank[e]
Endochitinase *win6.2C*	CN192741	grail3.0001024001	At3g12500	10^{-100}	**1**	**1**
Lipase, class 3	CN192786	estExt_Genewise1_v1.C_LG_IV2794	At4g18550	10^{-105}	**2**	**5**
Endochitinase *win8*	CN192595	estExt_fgenesh1_pg_v1.C_LG_IV1440	At3g12500	$5x10^{-87}$	**6**	**2**
Apyrase	xxxxxxxx	eugene3.00190357	At5g18280	10^{-116}	**4**	**6**
Vegetative storage protein *win4.5*	CN192930	eugene3.00130800	At4g24340	$3x10^{-49}$	**3**	**8**
Polyphenol oxidase *PtdPPO1*	CN193334	eugene3.00110805	At2g20590	0.14*	**10**	**3**
Unknown	CN192936	eugene3.00870012	At3g03150	$2x10^{-26}$	**5**	**9**
Kunitz trypsin inhibitor *PtdTI5*	CN192805	eugene3.00190800	At1g73325	$1x10^{-9}$	**--[8]**	**7**
Acid phosphatase, class B	CN193016	estExt_Genewise1_v1.C_LG_I0437	At4g25150	$2x10^{-67}$	**7**	**14**
Pop3 / SP1	xxxxxxxx	estExt_fgenesh1_pm_v1.C_LG_X0481	At3g17210	$4x10^{-21}$	**11**	**13**
Pop3- / SP1-like	xxxxxxxx	estExt_Genewise1_v1.C_LG_X0703	At3g17210	$6x10^{-18}$	**9**	**17**
Acyl-activating enzyme	CN192663	eugene3.00040736	At1g65890	0	**--[15]**	**12**
Kunitz trypsin inhibitor *PtdTI3*	CN192549	estExt_Genewise1_v1.C_LG_XIX2762	At1g73325	$2x10^{-9}$	**17**	**11**
Kunitz trypsin inhibitor *PtdTI4*	CN193330	eugene3.00040289	At1g17860	$4x10^{-28}$	**--[26]**	**4**
β-glucosidase	CN192799	estExt_fgenesh1_pm_v1.C_LG_X0568	At5g36890	0	**8**	**22**
β-amylase	CN192760	grail3.0064001202	At4g15210	0	**19**	**15**
Unknown	CN193014	estExt_fgenesh1_pg_v1.C_LG_XII0482	At2g37010	0.76*	**12**	**26**
Allene oxide cyclase	CN193019	eugene3.00040854	At1g13280	$2x10^{-69}$	**21**	**18**
Cytochrome P450	CN193274	eugene3.00030238	At5g07990	10^{-102}	**28**	**16**
Cytochrome P450	CN193412	eugene3.00030238	At5g07990	10^{-102}	**25**	**19**
18S rRNA gene	CN192944	xxxxxxxxxxxxxxxxxx	At3g41768	10^{-125}	**20**	**24**
Cytochrome P450	CN193236	eugene3.00030242	At5g07990	10^{-104}	**16**	**29**
Unknown protein (MOSC domain)	CN193222	grail3.0047000902	At1g30910	10^{-128}	**15**	**31**
Phenylalanine ammonia lyase	CN192894	estExt_Genewise1_v1.C_280661	At2g37040	0	**13**	**34**
Cytochrome P450	CN193273	eugene3.00280025	At5g07990	10^{-100}	**26**	**23**
Lipolytic enzyme, G-D-S-L	CN193295	eugene3.00121141	At5g45670	$3x10^{-79}$	**22**	**27**
Cinnamyl alcohol dehydrogenase	CN192800	estExt_fgenesh1_pm_v1.C_LG_II1065	At4g39330	10^{-112}	**14**	**39**
Esterase / Lipase	CN192875	eugene3.00090620	At3g48690	$2x10^{-59}$	**--[33]**	**20**
Unknown (DUF946 domain)	CN193384	eugene3.00180760	At2g44260	0	**24**	**37**
Cytochrome P450	CN193433	estExt_Genewise1_v1.C_LG_IV4159	At3g25180	10^{-144}	**27**	**35**
Pop3- / SP1-like	CN192903	estExt_fgenesh1_pm_v1.C_LG_X0482	At3g17210	$4x10^{-20}$	**29**	**41**
HVA22-related protein	CN192744	estExt_Genewise1_v1.C_LG_XII0452	At1g74520	$7x10^{-71}$	**18**	**52**
13-Lipoxygenase	CN192531	xxxxxxxxxxxxxxxxxx	At3g45140	$5x10^{-5}$	**--[49]**	**28**
Vegetative storage protein *pni288*	CN193425	eugene3.00190336	At4g24340	$2x10^{-39}$	**--**	**10**
Anthocyanidin synthase	CN192891	estExt_fgenesh1_pm_v1.C_LG_XVI0363	At5g05600	10^{-137}	**--**	**21**
ADP-glucose pyrophosphorylase	CN192812	eugene3.00120693	At1g74910	0	**23**	**--**
Neutral invertase	CN193364	estExt_Genewise1_v1.C_LG_VIII2120	At1g56560	0	**--**	**25**

[a]Genes significantly induced (two-fold induction, $P < 0.05$) by FTC-R or wounding were ranked by induction; the 30 most strongly FTC-R-induced genes and the 30 most strongly wound-induced genes were combined in a dually ranked list and shown.
[b]JGI gene model from the *P. trichocarpa* genome (http://genome.jgi-psf.org/Poptr1/Poptr1.home.html) that corresponds to the EST.
[c]AGI (Arabidopsis Genome Initiative) code for best match of poplar gene (JGI gene model) to *Arabidopsis thaliana* determined by BLASTX of TAIR (http://www.arabidopsis.org/). For 18S rRNA gene (CN192944), the *A. thaliana* gene that is most similar to the EST determined by BLASTN is shown.
[d]Expect value of best match of poplar gene to *A. thaliana* from BLASTX (BLASTN for rRNA gene). Asterisks indicate genes for which significant similarity were not found in the *A. thaliana* genome.
[e]Genes were ranked by level of induction. Genes with no ranking were not significantly induced. Superscript numbers denote ranking if the *P*-value is ignored; these genes were induced, but with a non-significant *P*-value.

χ^2 analysis, $P > 0.1$). This set of genes thus represents a substantial component of the herbivory-induced transcriptome. We found 24 ESTs corresponding to 17 unique genes with putative functions in primary metabolism that were strongly wound-induced (five-fold induction, $P < 0.05$; Table 2-2). Previous expression profiling of plant defense has suggested that the induction of primary metabolic genes is required for resource or nutrient reallocation during the defense response (Reymond et al., 2004). However, considering their strong induction by wounding and FTC-R, these primary metabolic genes could play more direct roles in defense (see Discussion). Among these genes was a group of carbohydrate-related enzymes, including a β-amylase, β-glucosidase, and neutral invertase. A second group of FTC-R- and wound-induced genes has putative functions in lipid metabolism, including several lipases and a gene belonging to the acyl-activating enzyme (AAE) superfamily, recently described for *Arabidopsis* (Shockey et al., 2003). Smith et al. (2004) had identified both of these types of lipid metabolism-related genes in a microarray analysis of abrasion wound-induced genes of poplar leaves, which supports the idea they may be of general importance. The gene represented by H1644 encodes a class 3 lipase and was extremely responsive to our simulated herbivory treatments, ranking second for FTC-R-induction and fifth for wound-induction (Table 2-2). Lipases belonging to the class 3 family have been previously identified with roles in defense signaling (see Discussion). We also identified genes encoding storage proteins that were FTC-R- and wound-induced. The wound-inducibility of two of these VSPs, *win4.5* and *pni288*, has been previously described (Davis et al., 1993; Lawrence et al., 2001). Our analysis also identified two wound-inducible genes encoding novel storage proteins annotated as 11S cupin and embryo-specific 3. The roles of wound-inducible storage proteins may be related to allocation of resources for defense, but their exact role is unknown (Christopher et al., 2004).

Seven highly wound- and FTC-R-inducible genes (five-fold induction, $P < 0.05$; Table 2-3) encoded proteins of unknown function. Two such unknowns (H241, H1958) are among the most strongly induced genes and showed the most abundant cellular transcripts in response to wounding or FTC-R (95th percentile in transcript abundance). Database searches for conserved domains revealed that three unknowns (H81, H522, H583; Table 2-3) contained the ZIM motif; this is a short motif found in many GATA-

Table 2-2. Macroarray data for selected induced genes with putative functions in primary metabolism.

Putative function	GenBank accession	JGI predicted gene name[a]	AGI accession[b]	E-value[c]	Induction factor[d]	
					Wound	FTC-R
Amino acid transport and metabolism						
Prephenate dehydratase	CN193183	eugene3.00660027	At1g08250	10^{-172}	**7.77**** ± 1.53	**1.58** ± 0.38
Carbohydrate transport and metabolism						
β-Amylase	CN192760	grail3.0064001202	At4g15210	0	**21.68**** ± 4.15	**3.40*** ± 1.02
β-Glucosidase	CN192799	estExt_fgenesh1_pm_v1.C_LG_X0568	At5g36890	0	**14.16**** ± 3.49	**5.67**** ± 1.17
Neutral invertase	CN193364	estExt_Genewise1_v1.C_LG_VIII2120	At1g56560	0	**12.00**** ± 1.73	**1.70*** ± 0.16
Glyoxalase	CN192888	estExt_Genewise1_v1.C_LG_IV1582	At1g11840	10^{-139}	**5.68**** ± 0.37	**1.78**** ± 0.07
UDP-Glucoronosyl/UDP-glucosyl transferase	CN192670	eugene3.00160092	At1g07250	10^{-106}	**7.05**** ± 1.70	**1.84** ± 0.43
Lipid transport and metabolism						
Lipase, class 3	CN192786	estExt_Genewise1_v1.C_LG_IV2794	At4g18550	10^{-105}	**97.79**** ± 26.44	**9.57**** ± 1.43
Acyl-activating enzyme	CN192663	eugene3.00040736	At1g65890	0	**25.52*** ± 20.08	**3.93** ± 2.46
Esterase / Lipase	CN192875	eugene3.00090620	At3g48690	$2x10^{-59}$	**14.56*** ± 7.22	**2.60** ± 0.77
Lipolytic enzyme, G-D-S-L	CN193295	eugene3.00121141	At5g45670	$3x10^{-79}$	**11.81**** ± 3.02	**2.74**** ± 0.16
Nucleotide transport and metabolism						
Apyrase	CN193208	eugene3.00190357	At5g18280	10^{-116}	**55.00**** ± 18.66	**6.84**** ± 1.76
Phosphatase						
Acid phosphatase, class B	CN193016	estExt_Genewise1_v1.C_LG_I0437	At4g25150	$2x10^{-67}$	**22.72**** ± 4.76	**6.66*** ± 2.56
Plant storage proteins						
Vegetative storage protein win4.5	CN192930	eugene3.00130800	At4g24340	$3x10^{-49}$	**55.41**** ± 19.13	**8.21**** ± 0.94
Vegetative storage protein pni288	CN193425	eugene3.00190336	At4g24340	$2x10^{-39}$	**30.36**** ± 4.77	**1.77** ± 0.67
11S Cupin plant seed storage protein	CN192638	eugene3.01070077	At1g07750	$2x10^{-88}$	**11.10**** ± 2.61	**1.97** ± 0.56
Embryo-specific 3 seed protein	CN192906	eugene3.00151111	At5g62200	$3x10^{-37}$	**9.87*** ± 6.03	**3.38** ± 1.12
Posttranslational modification, protein turnover, chaperones						
Glutathione S-transferase	CN192910	eugene3.00020134	At3g03190	$2x10^{-49}$	**8.83**** ± 2.08	**1.20** ± 0.15

[a]JGI gene model from the *P. trichocarpa* genome (http://genome.jgi-psf.org/Poptr1/Poptr1.home.html) that corresponds to the EST.
[b]AGI (Arabidopsis Genome Initiative) code for best match of poplar gene (JGI gene model) to *Arabidopsis thaliana* determined by BLASTX of TAIR (http://www.arabidopsis.org/).
[c]Expect value of best match of poplar gene to *A. thaliana* from BLASTX.
[d]Mean expression ratios (± SE) in wounded or FTC-R-treated leaves. Only selected genes with putative functions in primary metabolism and significant expression ratios of at least five-fold (P-value < 0.05) after plier wounding are shown. Asterisks denote the significance levels of treated plants compared to control plants (** - P-value < 0.01; * - P-value < 0.05).

Table 2-3. Macroarray data for selected induced genes with novel or unknown functions.

Clone ID	GenBank accession	JGI predicted gene name[a]	AGI accession[b]	E-value[c]	Conserved domain / motif[d]	Induction factor[e]	
						Wound	FTC-R
H1958	CN192936	eugene3.00870012	At3g03150	$2x10^{-26}$		**33.40**** ± 7.23	**6.86*** ± 2.08
H241	CN193014	estExt_fgenesh1_pg_v1.C_LG_XII0482	At2g37010	0.76		**11.93**** ± 3.28	**4.74*** ± 1.39
H66	CN193222	grail3.0047000902	At1g30910	10^{-28}	MOSC	**10.89**** ± 2.66	**3.55*** ± 1.18
H583	CN193162	estExt_Genewise1_v1.C_280164	At1g19180	$2x10^{-48}$	ZIM motif	**10.64**** ± 1.71	**1.58** ± 0.70
H892	CN193384	eugene3.00180760	At2g44260	0	DUF946	**8.68*** ± 2.39	**2.69*** ± 0.59
H1007	CN192515	fgenesh1_pg.C_LG_IX000909	CN192515	$1x10^{-118}$		**5.42**** ± 1.23	**0.98** ± 0.48
H604.12	CN193182	fgenesh1_pg.C_scaffold_166000056	At4g22290	$7x10^{-96}$		**5.13*** ± 1.59	**2.19** ± 0.67
H81	CN193314	grail3.0037000501	At1g19180	$2x10^{-31}$	ZIM motif	**4.48**** ± 0.83	**4.34** ± 2.27
H522	CN193114	estExt_fgenesh1_pg_v1.C_LG_VI0512	At5g20900	$7x10^{-29}$	ZIM motif	**4.27*** ± 1.20	**1.87** ± 0.62
H764	CN193276	estExt_fgenesh1_pg_v1.C_LG_I2037	At1g07050	$4x10^{-37}$	CCT motif	**4.09*** ± 0.81	**2.17** ± 1.21

[a]JGI gene model from the *P. trichocarpa* genome (http://genome.jgi-psf.org/Poptr1/Poptr1.home.html) that corresponds to the EST.
[b]AGI (Arabidopsis Genome Initiative) code for best match of poplar gene (JGI gene model) to *Arabidopsis thaliana* determined by BLASTX of TAIR (http://www.arabidopsis.org/).
[c]Expect value of best match of poplar gene to *A. thaliana* from BLASTX.
[d]Conserved domain / motif predicted by the conserved domain database (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi). Domain / motif accession numbers (pfam, InterPro): DUF946: PF06101, IPR009291; MOSC: PF03473, IPR005302; ZIM motif: pfam06200, IPR010399; CCT motif: pfam06203, IPR010402.
[e]Mean expression ratios (± SE) in wounded or FTC-R-treated leaves. Only selected genes with unknown functions and significant expression ratios of at least four-fold (P-value < 0.05) after plier wounding are shown. Asterisks denote the significance levels of treated plants compared to control plants (** - P-value < 0.01; * - P-value < 0.05).

type Zn-finger plant transcription factors. All three genes are significantly induced by wounding, and preliminary analysis of the kinetics of H583 induction indicated that expression is induced as early as 1 h (data not shown). This would be consistent with a role as a novel transcription factor involved in defense signaling (see Discussion).

2.4 Discussion

In order to characterize the hybrid poplar response to insect herbivory, we used macroarrays to investigate gene expression after treatment with FTC-R and mechanical wounding. Our experiments demonstrated that FTC-R-application induces a strong defense response in poplar leaves. Comparative macroarray analyses showed that severe mechanical wounding with pliers could elicit even higher expression levels and larger numbers of significantly induced genes. Moreover, our analyses showed that the response induced by FTC-R is a substantive subset of the wound-induced response, and that while the gene expression patterns between wound- and FTC-R-induction differed in intensity, they were qualitatively similar. In addition, we found extensively overlapping patterns of gene expression in local and systemic leaves, indicating a broad systemic response in poplar.

Induction of defenses by FTC-R and wounding

FTC-R is a potent inducer of herbivore defenses of poplar leaves, and it contains the FAC volicitin (*N*-hydroxylinolenoyl-L-glutamine; Fig. 2-1). The presence of volicitin in FTC-R suggests that it contributes to the elicitor activity of FTC-R. However, it is also possible that FTC-R contains additional elicitors, and the efficacy of volicitin in poplar will have to be verified directly. In other plants, regurgitant from a variety of caterpillar species has been shown to induce many plant responses elicited by herbivory, including defense gene expression in tobacco (Halitschke et al., 2001; Schittko et al., 2001; Halitschke et al., 2003; Roda et al., 2004) and volatile release in corn, cotton, maize, tobacco, and alfalfa (Alborn et al., 1997; Alborn et al., 2003; Halitschke et al., 2003; Roda et al., 2004). Thus, caterpillar regurgitant appears to be an excellent proxy for herbivory.

Both FTC-R and wounding induced strong defense responses, and in general, inducible genes present on the array were upregulated by both treatments. Moreover, we did not find any genes which were exclusively induced by either treatment (Fig. 2-5). It is possible that our array was simply too small to identify such elements; however, based on our EST analysis, it should include the most abundant wound-inducible genes. Thus, we tentatively conclude that the major elements of the wound response were to a large extent also inducible by FTC-R. This was initially surprising, since previous studies of gene expression and volatile release had suggested there are major differences between herbivore- and wound-induced responses (Alborn et al., 1997; McCloud and Baldwin, 1997; Halitschke et al., 2001; Halitschke et al., 2003; Reymond et al., 2004; Roda et al., 2004). However, how plants are wounded appears to have a major effect in demonstrating such differences, making it difficult to compare studies. Our wounding treatment was relatively severe and involved the crushing of leaf margins with pliers, repeated three times at 1 h intervals (see Methods). Previous studies have also damaged leaves by crushing (Reymond et al., 2000), but many use more subtle methods such as puncturing with fabric tracing wheels (Halitschke et al., 2003; Hui et al., 2003; Roda et al., 2004) and light abrasion (Smith et al., 2004). In addition to physically damaging leaf blades, some workers have mimicked feeding damage by removing leaf tissue using cork borers, scissors or even larval mandibles (McCloud and Baldwin, 1997; Schittko et al., 2001; Reymond et al., 2004). Significantly, these treatments all have different effects on gene expression. McCloud and Baldwin (1997) demonstrated that damage to tobacco plants caused by removing leaf tissue designed to mimic herbivory induces a lighter response than wounding with a tracing wheel. Moreover, a comparison of *Arabidopsis* transcript profiles comparing wounding of different intensities demonstrates that some genes induced by leaf crushing do not respond to leaf tissue removal (Reymond et al., 2000; Reymond et al., 2004). Finally, a recent study in lima bean using a "mechanical caterpillar" engineered to closely mimic insect feeding damage over an extended period found that both the spatial and temporal extent of physical damage affects the composition of released volatiles (Mithofer et al., 2005). Thus, the volatile profile previously thought to require live insects can be reproduced by accurately mimicking insect feeding behavior (Mithofer et al., 2005), which suggests that continuous damage is

a key component of damage perception by the plant. The repeated plier-wounding used for our study may therefore be sufficiently sustained and severe to be perceived as insect feeding, which is reflected by our inability to find significant differences in wound- and FTC-R-responses. By contrast, in our system light wounding with a tracing wheel had essentially no effect on upregulation of our genes in both macroarray and northern analyses (Fig. 2-4 and 2-5; data not shown). This treatment was therefore not useful for direct comparisons with FTC-R, and emphasizes that wounding intensity should be carefully controlled and may have different effects in different species.

We note that our comparisons were only carried out at a single (24h) time point, so that rapid and early differences in the kinetics of induction in response to FTC-R and wounding would have escaped detection. We chose the 24h time point since our previous studies indicated the induced expression of most poplar defense genes is sustained and maximal at 24 h after damage (Constabel et al., 2000; Christopher et al., 2004). Other expression profiling studies have shown more transient defense gene expression (Reymond et al., 2000; Cheong et al., 2002; Halitschke et al., 2003; Qu et al., 2004). Thus our inference that FTC-R and wounding by pliers cause qualitatively similar responses is preliminary; however, our experiments would have detected large-scale differences in gene expression profiles between FTC-R and wounding.

Our inability to find major differences between FTC-R and wounding in poplar leaves under our conditions suggests that FTC-R elicits a defense response at least in part by generating or mimicking an endogenous wound-signal. How volicitin and insect elicitors interact with cellular signaling pathways to induce defense responses is still unclear. Volicitin from regurgitant was shown to enter leaves during feeding by *S. exigua* (Truitt and Pare, 2004), and a putative receptor for volicitin has been identified in plasma membrane fractions of *Zea mays* (Truitt et al., 2004). Volicitin and other FACs may interact with JA signaling since regurgitant from *M. sexta* amplifies wound-induced increases in JA in tobacco and maize (McCloud and Baldwin, 1997; Halitschke et al., 2001; Schmelz et al., 2003; Roda et al., 2004). Given the central role of JA in wound-responses, increased JA levels could explain the elevated systemic responses elicited by insect regurgitants. How FACs interact with endogenous jasmonates and other signals

will become more apparent as the complexity of these stress signals is dissected further (Howe, 2004).

A secondary aim of our study was to determine if the defense response differed in systemically induced and directly damaged or treated leaves. Some reports have found distinct differences in local versus systemically induced gene expression; for example, in *Arabidopsis* preferential expression of some genes in either local or systemic tissues is detected (Titarenko et al., 1997; Rojo et al., 1999). In tomato, expression of locally-induced octadecanoid pathway genes, and subsequent accumulation of JA, are barely detected in systemic tissues (Strassner et al., 2002). In contrast, an extensive overlap was demonstrated between local and systemic responses of *Arabidopsis* challenged by *P. rapae* (Reymond et al., 2004). Our current macroarray experiments failed to detect significant qualitative differences between damaged and systemic leaves, although there were quantitative differences (Fig. 2-6). Again, due to the modest size of the array some differentially expressed genes might have been missed. However, the similarity in expression does suggest that the poplar defense response is very similar in systemic and wounded leaves, although in previous work we also found some differences in these responses (Christopher et al., 2004). This discrepancy could be due to different plants modulating systemic defenses differently, possibly due to age effects in systemic leaves. In this study, we minimized differences due to leaf age and development by selecting three comparable leaves, which were pooled for analysis to minimize individual leaf variability. As mentioned, we only investigated responses at a single time point of 24 h after treatment, and discrepancies with our previous data may be attributable to differences in kinetics. Nevertheless, our result is consistent with current ideas of systemic defense signaling, where wounding activates a signal cascade that is suggested to amplify a local defense signal that then undergoes long-distance transport to systemic tissues and induces the defense response (Ryan and Moura, 2002; Howe, 2004). This would predict little or no attenuation of the wound-signal; the strong systemic response following wounding we have detected is likely the result of such a signal cascade linking damaged and systemic leaves. This defense system would ensure induced resistance in undamaged leaves and thereby reduce overall damage by feeding insects.

Many induced poplar genes encode enzymes of secondary and primary metabolism

Transcript profiling studies in *Arabidopsis* indicate that dozens to hundreds of genes can be induced by physical and insect damage (Cheong et al., 2002; Reymond et al., 2004), and estimates from tobacco indicate that at least 500 genes are affected by herbivory (Hermsmeier et al., 2001). In hybrid poplar, 9% of genes on a 10,000 gene array were found to be induced 14 days after abrasion damage (Smith et al., 2004). Recently, Ralph et al. (2006a) reported that 1191 genes on a 15,500 gene array showed upregulation 24h after FTC-herbivory of *P. trichocarpa* × *P. deltoides.* Our more specialized and smaller macroarray analysis identified FTC-R- and wound-induction of approximately 100 unique genes, with both known and unknown functions, which together constituted about 20% of the genes on our macroarrays (Fig. 2-2). As expected, among the inducible genes we found a significant representation of genes known to be involved in defense, including Kunitz trypsin inhibitors, polyphenol oxidase, chitinase, as well as cytochromes P450 and enzymes of secondary metabolism. However, we also found that a substantial number of genes predicted to encode enzymes of primary metabolism are strongly induced.

Our analysis indicated that genes implicated in carbohydrate and lipid metabolism, and those encoding VSPs, are prevalent among the highly induced genes (Tables 2-1 and 2-2). Previous workers have noted that herbivory stress can result in suppression of genes involved in important metabolic processes such as photosynthesis. This has led to the suggestion that altered expression of primary metabolic genes is a reflection of a shift of resources to defense (Reymond et al., 2004; Voelckel and Baldwin, 2004). The systemic upregulation of cell wall invertase during herbivore defense in poplar has been associated with increased sink strength and a concomitant enhancement of carbohydrate import and condensed tannin accumulation (Arnold and Schultz, 2002). Such an increase in sink strength could be indicative of resource allocation for defense in young leaves. Interestingly, it would also have implications for systemic signaling, since systemic signaling in poplar is dependent on phloem transport and moves preferentially from source to sink (Davis et al., 1991b; Arnold and Schultz, 2002). Whether the neutral invertase identified by our array analysis has a similar function is unclear; however, this provides a starting point for future studies on the reallocation of resources during defense.

Other enzymes identified by our analysis, such as a putative class 3 lipase, esterase, and AAE are involved in lipid metabolism (Tables 2-1 and 2-2). Significantly, class 3 lipases were previously identified in *Arabidopsis* with roles in pathogen and insect defense. For example, the *PAD4* and *EDS1* genes encode class 3 lipase proteins that are involved in salicylic acid accumulation during pathogen defense (Falk et al., 1999; Jirage et al., 1999). A distinct class 3 lipase from *Arabidopsis*, *DAD1* (defective in anther dehiscence), has phospholipase A_1 activity and catalyzes the initial step of JA biosynthesis by releasing linolenic acid from chloroplast membrane phospholipids (Ishiguro et al., 2001). However, DAD1-catalyzed JA accumulation is required for floral development in *Arabidopsis*, but not for herbivore-defense (Schaller et al., 2004). Instead, another of the 12 *Arabidopsis DAD1*-related genes, which includes the putative ortholog of our lipase, appears to be involved in JA-based defense signaling (Beisson et al., 2003). JA metabolism could also provide a rationale for the induction of a putative AAE, which belongs to a larger superfamily of AAEs (Shockey et al., 2003). *JAR1,* which conjugates isoleucine to JA, is a member of this family that is required for many JA responses (Staswick et al., 2002; Staswick and Tiryaki, 2004). While we do not know if our class 3 lipase and the AAE genes are directly involved in JA metabolism, they provide potential links between fatty acid metabolism and herbivore defense to be investigated further.

Several unknown genes containing the ZIM motif are induced during poplar defense

Detailed bioinformatic analysis identified three unknown genes with a ZIM motif. The function of the ZIM motif is unknown, but it has been suggested to be involved in DNA binding (CDD, pfam06200; InterPro, IPR010399). The ZIM (*Zn-finger protein expressed in Inflorescence Meristem*) protein is an *Arabidopsis* transcription factor with a putative role in development, and has discrete domains responsible for DNA binding, transcriptional activation, putative protein-protein interactions, and nuclear localization (Nishii et al., 2000; Shikata et al., 2003; Shikata et al., 2004). All three of our genes with the ZIM motif lack the DNA binding GATA-type Zn-finger domain, but it is possible that they activate transcription through an interacting partner. Shitaka et al. (2003) suggested that *Arabidopsis* ZIM requires an interacting partner for full-strength transcriptional activation. Our ZIM motif genes also each contain an N-terminus rich in

acidic residues, which has transcriptional activation activity in *Arabidopsis* (Shikata et al., 2003). Finally, these genes contain a region rich in basic residues with several conserved basic residues to the probable nuclear localization signal (NLS) of *Arabidopsis* ZIM (Nishii et al., 2000). A predicted NLS was not detected from any of our genes using available search tools (PredictNLS, Prosite), but these tools also failed to detect an NLS from *Arabidopsis* ZIM. Additional experiments are required to establish whether our ZIM motif genes are localized to the nucleus or active as transcriptional activators.

2.5 Conclusion

The expression profiling approach we have described in this report has allowed us to get a first glimpse of poplar defense responses induced by an insect elicitor compared to mechanical wounding. We have shown that FTC-R is a potent elicitor of defense responses in poplar, and that for our suite of genes, the FTC-R- and wound-induced responses were qualitatively similar while quantitatively distinct. The availability of larger poplar microarrays will help test this result; if corroborated, this may reflect a general defense strategy which evolved in perennial plants, or the effect of a response elicited by a polyphagous generalist. The large number of natural poplar pests will facilitate comparative experiments with herbivores having differing host ranges and allow a unique molecular understanding of plant defense. Poplars are keystone species of the boreal forest, and both *Populus* and *Malacosoma* species are found throughout the Northern hemisphere. With the recent completion of the poplar genome sequence, the poplar-FTC system presents an excellent opportunity for molecular and ecological studies of a plant-herbivore interaction that is relevant on a global scale.

3 Insect regurgitant and wounding elicit similar defense responses in poplar leaves: Not something to spit at?

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3.1 Introduction

Plants have evolved sophisticated adaptive responses to herbivory, including physical and biochemical, constitutive and inducible defense strategies. The induction of defenses implies there are sensitive mechanisms to perceive and transduce herbivory into a coordinated defense response. A variety of evidence suggests that plants interpret diverse stimuli from feeding insects, including physical wounding, insect-derived elicitors in saliva, and metabolites released from damaged cells. In particular, a number of studies have now shown that insect regurgitant can effectively mimic live insects as inducers of plant defense.

3.2 Results and Discussion

We recently used macroarrays to compare transcript profiles in hybrid poplar (*Populus trichocarpa* × *P. deltoides*) leaves elicited by wounding and by regurgitant from forest tent caterpillar, a poplar defoliator (FTC-R; *Malacosoma disstria*). Both treatments upregulate a large number of genes, many of which encode known and suspected anti-herbivore proteins (Chapter 2). For the set of genes represented on our array, the effects of these treatments differed only quantitatively (the wound response being stronger), and we detected no significant qualitative differences or insect-specific induction. This finding was somewhat surprising, since recent reports in other systems had demonstrated that insect herbivory can have effects that are quite distinct from wounding (Reymond et al., 2004; Roda et al., 2004; De Vos et al., 2006). However, in comparison to these studies, our wound treatment was much more severe; we wounded plants by crushing leaf margins with pliers rather than the commonly used leaf puncture method. The wounding treatment was applied three times at hourly intervals, which together resulted in the

necrosis of approximately 25% of total leaf area. We suggest that both the extent of damage and the repetitive nature of our treatment are responsible for the strong induction of gene expression that we observed, since a recent study using a 'mechanical caterpillar' found that both the spatial and temporal pattern of wounding is key in shaping the defense response (Mithofer et al., 2005). Therefore, differences in wounding protocols used to simulate insect feeding (i.e. crushing, puncturing, abrading, and tissue removal) can complicate direct comparisons of different studies.

Since our comparisons were carried out at a single time point (24 h) and we used macroarrays with a relatively small number of genes from a wound-induced library, we cannot rule out the possibility that FTC-R induces insect-specific genes that we failed to detect. Nevertheless, our findings do suggest that wound and caterpillar regurgitant responses can be more similar than previously thought. This result may be particularly relevant for long-lived perennials such as poplar, which may deploy a broad response to generalists such as FTC. Interestingly, another tree species, Sitka spruce (*Picea sitchensis*), also showed considerable overlap in its responses to wounding and weevil or budworm herbivory (boring and chewing insects, respectively)(Ralph et al., 2006b).

We carried out additional experiments to gain further insights into the overlap between wound- and insect-responses. To determine whether FTC-R could reshape the response induced by intense wounding, we applied FTC-R to plier wounds (Fig. 3-1). The FTC-R only marginally enhanced the wound response; using *PtdTI3* (a Kunitz trypsin inhibitor) as a marker gene, an increase of approx. 14% in transcript abundance was measured. By contrast, treatment of leaf punctures with FTC-R increases transcripts levels of *PtdTI3* by approx. 300% (Chapter 2). This confirms that our wound-induction treatment is intense and suggests it triggers a near-maximal response.

To ascertain the potential signaling role of jasmonates (JAs) in these responses, we compared the FTC-R- and wound-induced gene sets with macroarray data from an analogous methyl-jasmonate (MeJA) induction experiment. A large proportion of the genes (84%) that were induced by both wounding and FTC-R were also MeJA-inducible. Furthermore, the ranked list of the most induced genes is similar for all three treatments; for example seven of the top ten genes for each treatment are common (Table 3-1). Since

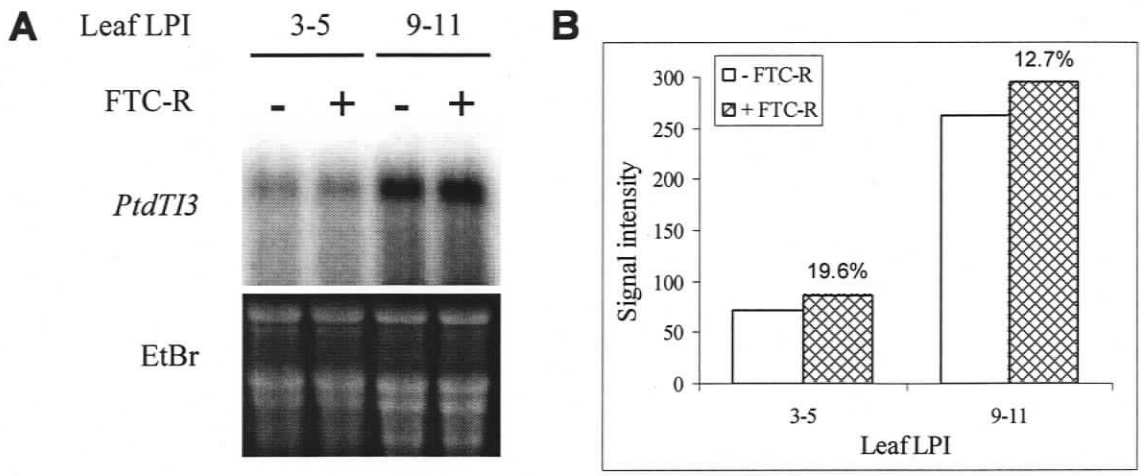


Figure 3-1. Accumulation of *PtdTI3* (Kunitz trypsin inhibitor 3) mRNA in leaves of hybrid poplar wounded with pliers and treated with (+) or without (-) forest tent caterpillar regurgitant (FTC-R).

A. Leaves of 9 – 11 were wounded with pliers and mock treated with ddH_2O or a 1:5 dilution (v/v with ddH_2O) of FTC-R treated three times, at 1-h intervals, and leaves 9-11 and 3-5 (local and systemic responses, respectively) were harvested 24-h after start of the treatment and analyzed by northern blot analysis. The experiment was replicated once with the same results. Northern analysis was performed and quantified as described (Chapter 2).

B. Quantified transcript abundance from (A). Open bars are mock-treated control plants and hatched bars are FTC-R-treated plants. Percentages above hatched bars show the increase in transcript abundance by FTC-R treatment.

Table 3-1. Comparison of relative MeJA-, FTC-R- and wound-induction rankings of the most strongly MeJA-induced genes from macroarray analyses[a]

Putative function	GenBank accession	FTC-R rank	Wound rank	MeJA rank
Kunitz trypsin inhibitor *PtdTI4*	CN193330	26[b]	**4**	**1**
Endochitinase *win6.2C*	CN192741	**1**	**1**	**2**
Lipase, class 3	CN192786	**2**	**5**	**3**
Polyphenol oxidase *PtdPPO1*	CN193334	**10**	**3**	**4**
Endochitinase *win8*	CN192595	**6**	**2**	**5**
Kunitz trypsin inhibitor *PtdTI3*	CN192549	**17**	**11**	**6**
Apyrase	xxxxxxxx	**4**	**6**	**7**
Vegetative storage protein *win4.5*	CN192930	**3**	**8**	**8**
Unknown	CN192936	**5**	**9**	**9**
Pop3 / SP1	xxxxxxxx	**11**	**13**	**10**

[a]Genes significantly induced (two-fold induction, $P < 0.05$ as measured by a Student's *t*-test) by methyl-jasmonate (MeJA), forest tent caterpillar regurgitant (FTC-R) or wounding were ranked by induction; the ten most strongly MeJA-induced genes are shown with the relative induction rankings by FTC-R and wounding of our previous study (Chapter 2). MeJA treatment of hybrid poplar was performed as described previously (Constabel et al., 2000), except that MeJA-treated plants were compared to untreated, control plants . Macroarray analysis of MeJA-treatment was performed as described previously (Chapter 2).
[b]*PtdTI4* was induced by FTC-R treatment, but with a non-significant *P*-value. Superscript number denotes induction ranking if the *P*-value is ignored.

JA and its derivatives play key roles in herbivore defense signaling (Schilmiller and Howe, 2005), this suggests that a common JA signaling pathway activates both FTC-R- and wound-induced responses. The extent of participation of the JA pathway in FTC-R- and wound-induced responses will have to be tested more directly, for example by generating a poplar *coi1* knockout deficient in JA signaling. Analogous experiments in *Arabidopsis* have identified JA-dependent as well as independent pathways involved in insect and wound-induced responses (Reymond et al., 2004).

How FTC-R and wounding can lead to a common induction of JA signaling and subsequent downstream responses is not clear. One possibility involves plant-derived elicitor compounds, which are released from damaged plant tissues following wounding and should thus be present in caterpillar regurgitant. We noted that our plier wound treatment leads to large necrotic areas on the damaged leaf, which would be a substantial source of such elicitors. Examples of known plant-derived elicitors include oligogalacturonides, the oligopeptides systemin and inceptin, hydrogen peroxide, and ATP (Gatehouse, 2002; Schmelz et al., 2006; Song et al., 2006). Furthermore, we found that treatments which cause necrosis via other stresses (e.g., high NaCl) also induce expression of the *PtdTI3* gene (Major and Constabel, unpublished data). This is unlikely to reflect a general stress response, since *PtdTI3* has been shown to encode a functional trypsin inhibitor with anti-herbivore properties. Other studies have also found an overlap in responses to wounding and abiotic stress (Cheong et al., 2002; Swindell, 2006), and tomato leaves are known to accumulate proteinase inhibitors following salt stress (Dombrowski, 2003). We speculate that large necrotic areas can release compounds that effectively elicit defense responses. Potent elicitors identified from insect regurgitant, such as fatty acid-amino acid conjugates (FACs) and peptide inceptin, are known to originate from plant fatty acids and proteins, respectively. Insects may thus process and perhaps concentrate molecules of plant origin during feeding, thereby increasing their potency as defense elicitors in regurgitant.

We note that our model does not preclude insect-specific responses; insect-specific modification of elicitors may reshape the plant response for any given plant-insect interaction. Likewise, salivary factors such as glucose oxidase (Musser et al., 2005) may suppress some elements of the general plant response. Nevertheless, our data are

consistent with the view that plants have evolved a strategy of defense induction based primarily on the recognition of tissue damage.

4 Shoot-root defense signaling and activation of root defense by leaf damage in poplar

[The following chapter has been submitted for a special *Populus* issue of the Canadian Journal of Botany]

4.1 Introduction

Plants respond to challenge by herbivores with inducible defenses that have been extensively documented in aerial tissues such as shoots and leaves, and their effects studied on aboveground pests. By contrast, the effects of belowground pests and the inducible root defenses are often ignored, even though roots constitute a significant component of plant biomass and are a food source for a substantial number of pests (Blossey and Hunt-Joshi, 2003; Schoonhoven et al., 2005). Root herbivores can affect a variety of physiological processes, including uptake of water, nutrients and minerals, carbohydrate storage, and production of hormones and phytochemicals. Studies of aboveground and belowground herbivore feeding have shown that feeding on either shoots or roots can enhance herbivore resistance in both organs. For example, rice defoliation by fall armyworm (*Spodoptera frugiperda*) reduces growth rates of rice water weevil (*Lissorhoptrus oryzophilus*), and likewise, root feeding by rice water weevil reduces growth rates of fall armyworm (Tindall and Stout, 2001). However, only recently have shoot-root defense interactions been investigated in the context of reciprocal plant resistance between aboveground and belowground herbivores, as these negative effects are often interpreted as a reduction of plant biomass and nutritional quality.

Inducible defenses against herbivores include toxins, antifeedants and antinutrients. For example, many plants synthesize antinutritive proteinase inhibitor proteins (PIs), which inhibit insect digestive enzymes, in response to herbivory (Ryan, 1990). Plant PIs are often present as multigene families from several nonhomologous types that inhibit all four mechanistic classes of proteinases, thereby conferring resistance against a broad range of phytophagous pests. In some species, the defense arsenal includes secondary metabolites such as alkaloids, terpenoids, glucosinolates and phenolics (reviewed by Kessler and Baldwin 2002). These types of defenses can also be

induced in roots. For example, in *Brassica nigra*, challenge by cabbage root fly (*Delia radicum*) induces antifeedant glucosinolate levels in roots (van Dam and Raaijmakers, 2006). In cotton, wireworm (*Agriotes lineatus*) root feeding increases terpenoid levels of roots (Bezemer et al., 2004). Interestingly, in both cases root herbivory also induces a systemic increase in the leaves, illustrating a root-shoot systemic defense response. Recently, indirect inducible defense was shown for maize roots, which in response to feeding by western corn rootworm (*Diabrotica virgifera vergifera*), release the volatile (*E*)-β-caryophyllene which diffuses through the soil and attracts the entomopathogenic nematode *Heterorhabditis megidis* (Rasmann et al., 2005).

Similarily, induction of root defenses by leaf treatment has been observed. For example, in *B. rapa*, glucosinolate levels in roots increase after foliar methyl jasmonate (MJ) treatment (Loivamaki et al., 2004). In *N. attenuata*, simulated herbivory of leaves increases levels of nicotine and proteinase inhibitor activity in roots (van Dam et al., 2001; Kessler and Baldwin, 2002). This induction of defenses in roots implies the transport of a systemic defense signal from the shoot to the roots. Downward, or basipetal systemic responses have only been reported in a few systems such as potato (Pena-Cortes et al., 1988), tobacco (Schittko and Baldwin, 2003) and poplar (Jones et al., 1993). Current understanding of systemic signaling suggests that a jasmonic acid signal (JA or JA derivative) generated in response to herbivory is transported via phloem and is responsible for inducing systemic defense responses (Schilmiller and Howe, 2005). However, basipetal signaling is often ignored by investigations of the systemic defense signal, which focus on upward or acropetal signaling. The basipetal systemic signal responsible for inducing root responses may also be JA, since leaf damage increases root JA pools and JA is directly transported from leaves to roots of tobacco (Baldwin et al., 1994; Zhang and Baldwin, 1997). Moreover, foliar application of JA has been shown to increase root herbivore resistance. For example, JA applied to leaves increases resistance against root-feeding grape phylloxera (*Daktulosphaira vitifoliae*) in *Vitis vinifera* (Omer et al., 2000) and root-knot nematodes (*Meloidogyne incognita*) in tomato (Cooper et al., 2005).

Few studies have investigated inducible root defense responses from a molecular perspective, although Baldwin and co-workers showed that folivore feeding generates a

systemic signal that induces the expression of putrescine *N*-methyltransferase mRNA in roots of *Nicotiana attenuata* (Winz and Baldwin, 2001). This gene encodes a key regulatory enzyme for nicotine biosynthesis, and its induction leads to production of nicotine in roots, which is subsequently transported to leaves. This demonstrates that plants can respond to aboveground pest attack with active regulation of gene expression in roots, but it is not clear whether the increased nicotine levels in roots provide enhanced resistance to belowground pests. However, protein-based defenses have been shown to have negative effects on root pests. Transgenic overexpression of sporamin (a serine proteinase inhibitor) in sugar beet and of oryzacystatin (a cysteine proteinase inhibitor) in *Arabidopsis* increases resistance against nematodes (Urwin et al., 2000; Cai et al., 2003).

In poplar, established inducible defense genes include Kunitz trypsin inhibitors (KTIs), chitinases and polyphenol oxidases (PPO) (Parsons et al., 1989; Davis et al., 1991a; Constabel et al., 2000; Lawrence and Novak, 2001; Wang and Constabel, 2004a; Lawrence and Novak, 2006). The list of known herbivore-inducible poplar genes has expanded rapidly with transcript profiling studies (Chapter 2; Christopher et al., 2004; Lawrence et al., 2006; Ralph et al., 2006a), which highlight the application of genomics to the analysis of inducible defense responses. We recently employed macroarrays to profile the transcriptional changes of hybrid poplar (*Populus trichocarpa* × *P. deltoides*) triggered by wounding or by an insect-elicitor of forest tent caterpillar (FTC; *Malacosoma disstria*) (Chapter 2). Comparison of local and systemic leaf responses indicated an extensive overlap, and we suggested that a strong systemic response would ensure induced resistance in undamaged leaves and reduce overall damage by feeding insects. Here, we extend the study of poplar systemic defense induction with an investigation of shoot-root systemic signaling. We present evidence for a basipetal systemic defense signal and a systemically induced root response, and compare and contrast the responses in leaves and roots.

4.2 Materials and Methods

Plant material

Poplar hybrid H11-11 saplings (*Populus trichocarpa* × *P. deltoides*), originating from the University of Washington/Washington State University Poplar Research

Program, were propagated from greenwood cuttings in Sunshine Mix #4 (Sungro, Seba Beach, AB, Canada) in 0.25 L propagation containers (RootMaker, Huntsville, AL, USA). After plantlets had rooted and reached a height of approximately 10 cm, they were transplanted to 15 cm-diameter pots containing Sunshine Mix #4 plus slow-release nutrients (8.9 g L^{-1} controlled release 8-6-12 NPK plus micronutrients (Acer, Delta, BC, Canada), 0.458 g L^{-1} superphosphate 0–20–0 (Green Valley, Surrey, BC, Canada), 1.21 g L^{-1} Micromax Micronutrients (Scotts-Sierra, Marysville, OH, USA), and 4.75 g L^{-1} Dolomite lime (IMASCO, Surrey, BC, Canada)). Plants were maintained in the Bev Glover Greenhouse at the University of Victoria. Supplemental lighting from 600 W high pressure sodium lamps was used to extend the photoperiod to 16/8 hr, and the temperature within the greenhouse was maintained at 25/18°C. Plants were watered daily with a solution containing 0.1 g L^{-1} 20-20-20 PlantProd fertilizer (Plant Products, Brampton, ON, Canada). All lateral shoots were pruned as they developed so that each plant consisted of a single main stem, no less than 2 weeks prior to experiments.

Methyl jasmonate (MJ) and wounding treatments

Plants were 12 weeks old and 1 m tall with approximately 30 leaves when used for experiments. MJ (Bedoukian Research, Danbury, CT) was diluted 1:10 with 95% (v/v) ethanol, and then rediluted 1:500 with water:0.1% (v/v) Triton X-100 for treatment. Controls for MJ treatment were untreated saplings because saplings mock treated with the same solution without MJ exhibited a low level induction, presumably due to Triton X-100 in the solution (data not shown). Shoots were treated by spraying leaves to the point of runoff, three times, at 1 h-intervals. Roots were treated by adding the MJ-ethanol solution to fertilizer solution in a 1:5000 dilution. Similar JA treatment of shoots and roots is reported to induce responses in roots of other plants (Baldwin et al., 1994; van Dam et al., 2004; Cooper et al., 2005). For mechanical wounding, leaf margins were wounded by crushing with pliers, three times, at 1 h-intervals. We have shown that this wounding method elicits a strong defense response (Chapter 2). Leaves were numbered using the Leaf Plastochron Index (Larson and Isebrands, 1971), with the index leaf (LPI 0) defined as the first developing leaf with a lamina length of 20 mm. For studying transcriptional changes, leaves of LPI 9-17 were wounded and tissue collected 24-h after

start of treatment. For studying changes of protein levels and activity, all unfolded leaves (LPI > 0) were wounded and tissue collected 4-d after start of treatment. Unless otherwise indicated, root samples consisted of the root crown (main root, <10 cm from soil surface). Immediately after harvesting, tissues were frozen in liquid nitrogen, and stored at -80°C until analyzed.

RNA extraction and hybridization

Total RNA was isolated from hybrid poplar leaves, quantified by UV absorbance, and quality verified on ethidium bromide (EtBr)-stained agarose gels as previously described (Haruta et al., 2001a). RNA (10 μg per lane) was loaded onto 1.2% (w/v) agarose-formaldehyde gels, and blotted overnight onto Hybond-N^+ nylon membranes (GE Healthcare Bio-sciences, Baie d'Urfé, PQ, Canada). RNA blots were probed with cDNA clones labeled with [α-^{32}P]dCTP (Rediprime II kit, GE Healthcare). Hybridizations were performed at 65°C and were washed at high stringency according to Church and Gilbert (1984). The blots were detected with a Storm PhosphorImager (GE Healthcare) and signal intensities were quantified using ImageQuant (GE Healthcare). EtBr staining of RNA was used to verify equal loading of lanes and EtBr-stained 25S rRNA bands were used to normalize quantified signal intensities.

Protein extraction, western blot detection and TI activity assays

Protein was extracted with Na_2HPO_4 buffer (100 mM, pH 7.0) containing 0.1% Triton X-100, 5% w/v polyvinylpolypyrrolidone, and 1% 2-mercaptoethanol. Extracts were clarified by centrifugation, and soluble protein quantified using the method of Bradford (1976). For western blotting, proteins were separated by SDS-PAGE and electro-transferred onto PVDF membranes (Pierce, Fisher Canada, Nepean, ON, Canada), and Ponceau S (Sigma) staining was used to verify equal loading and transfer efficiency. Western blot detection was carried out using polyclonal antibodies raised against poplar PPO (Wang and Constabel, 2004b), TI2 (Haruta et al., 2001a), VSP WIN4 (Lawrence et al., 1997), and TI3 and Pop3-like (Major and Constabel, unpublished data). Immunocomplexes were detected using acid phosphatase- or horseradish peroxidase-conjugated secondary antibodies (Bio-Rad, Hercules, CA, USA) and blots were

developed colourimetrically with reagents 5-bromo-4-chloro-3-indoyl phosphate (BCIP, Pierce) and nitroblue tetrazolium chloride (NBT, Pierce; acid phosphatase) or 3,3'-diaminobenzidine tetrahydrochloride (DAB, Sigma; horseradish peroxidase). Root protein accumulation was quantified from six distinct leaf wounding experiments (six control saplings paired with six wounded saplings) using ImageQuant (GE Healthcare) to detect bands from blots scanned at 300 dpi (Hewlett-Packard Scanjet 3670), and the ratio of leaf wounded to control was used to calculate average fold induction. PI activity was determined by titrating crude protein extracts with a standard quantity of bovine trypsin (Sigma), and measuring residual trypsin activity as the change of A_{247}/min due to cleavage of the trypsin substrate TAME (p-toluene-sulfonyl-L-arginine methyl ester, Sigma) as described by Worthington (1988). PI activity of root extracts was determined against trypsin, since strong inhibitory activity from leaf extracts and poplar TI3 is observed against trypsin (Major and Constabel, unpublished data). Percent trypsin inhibition was plotted against the square of root extract protein (μg). For statistical comparison of trypsin inhibitory activity in roots of control and leaf wounding treatments, we compared slopes (% inhibition/(μg protein extract)^{½}) from linear regression analysis and calculated a *P*-value (two-tailed) testing the null hypothesis that the slopes are identical (TI activity is equal from control and wound treatments).

Macroarray analysis

Macroarray analysis was performed as described previously (Chapter 2). Macroarrays were constructed from 580 cDNA inserts generated from systemic leaves of wounded (569 cDNAs; Christopher et al., 2004) and forest tent caterpillar (*Malacosoma disstria*) challenged (11 cDNAs; Patton and Constabel, unpublished) hybrid poplar saplings. Briefly for array analysis, total RNA was isolated from roots of three independent biological replicates for wounding and MJ treatments, as well as the corresponding controls for each. Each replicate was analyzed on an individual macroarray. Macroarrays were hybridized with ^{33}P-labelled target cDNA at 65°C and washed at high stringency according to Church and Gilbert (1984). Exposed PhosphoImager screens were scanned with a Storm PhosphoImager (GE Healthcare) and the signals were quantified using ArrayVision 7.0 (Imaging Research, St. Catherines, ON,

Canada). Background-corrected spot intensities were normalized to the standard deviation of the entire array (Richmond and Somerville, 2000). Normalized intensities from the three biological replicates were used to calculate average expression ratios and a Student's *t*-test (paired, one-tailed) of $\log_2$-transformed data was used to determine statistical significance. Because the macroarrays were constructed with cDNAs derived from leaves, we removed genes with very low expression levels. We selected a threshold for reliable expression of 35% of the average transcript abundance of control roots. This threshold was selected because it maximized removal of genes with functions in photosynthesis (Christopher et al., 2004) that are not expressed in non-photosynthetic tissues such as roots, while minimizing the removal of genes that were significantly up-regulated. To provide some corroborating evidence that the genes we excluded are actually not expressed in roots, we queried databases containing ESTs generated from root tissues, including PopulusDB (http://poppel.fysbot.umu.se/; Sterky et al., 2004), PoplarDB (http://mycor.nancy.inra.fr/PoplarDB/index.html; Kohler et al., 2003), and The Gene Index Databases (Quackenbush et al., 2001). The *in silico* analysis suggested that our threshold is conservative and that some root-expressed genes were likely excluded. However, our 35% threshold greatly reduced the proportion of false positives (genes identified as induced by the array but without root expression support in the databases); considering the composition of the arrays, the reduction of false positives was a priority. To create a non-redundant set of genes induced by our treatments in roots, we queried the JGI poplar genome database (http://genome.jgi-psf.org/Poptr1/Poptr1.home.html) and obtained full-length sequences and JGI accessions for each candidate gene.

4.3 Results

Methyl-jasmonate induces acropetal and basipetal shoot-root signaling

Our previous work on poplar leaves demonstrated that simulated herbivory by mechanical wounding or application of insect regurgitant from forest tent caterpillar (*Malacosoma disstria*) induced strong transcriptional changes in treated leaves and untreated (systemic) leaves on treated plants (Chapter 2). To investigate the possibility of systemic defense signaling between shoots and roots, we treated poplar shoots or roots with methyl-jasmonate (MJ) in experiments designed to test reciprocal induction. Shoots

were treated by spraying leaves three times with MJ at 1-h intervals, and roots were treated by supplementing water with MJ over a 24-h period. Both plant organs were then assayed for an inducible defense response by monitoring expression of poplar trypsin inhibitor 3 (*PtdTI3*), since we have previously shown it is an excellent defense marker (Chapter 2) and it encodes a functional proteinase inhibitor with anti-insect properties (Major and Constabel, unpublished data). MJ applied to roots by irrigation resulted in a strong induction of *PtdTI3* mRNA in roots, as well as a moderate response in leaves (Fig. 4-1 A,B); the average fold changes in the roots and leaves from four replicate experiments were 43X and 6.6X, respectively. This systemic response implies the movement of an acropetal (upwardly mobile) systemic defense signal. Likewise, application of MJ to shoots induced *PtdTI3* mRNA not only in leaves, but also in the roots (Fig. 4-1 C,D). In this case, leaf and root tissues responded equally to shoot treatment, since the average fold changes in the leaves and roots from three replicate experiments was 9.4X and 8.9X, respectively. These results imply the induction of a basipetal (downward) systemic signal.

Mechanical wounding induces basipetal shoot-root signaling

Since exogenous application of MJ to plants can be hydrolyzed to JA, which may be transported to leaves and roots (Zhang and Baldwin, 1997), it is not clear whether application of MJ induces a *de novo* systemic response. To determine if bi-directional systemic defense signaling can be induced in the absence of direct MJ application, we wounded leaves of LPI 9-17 with pliers, and measured levels of *PtdTI3* mRNA in unwounded tissues above and below the wounded leaves using northern blots (Fig. 4-2). Transcripts of *PtdTI3* were induced in the apical bud and leaves 3-5, indicating an acropetal systemic response, as previously shown for poplar (Davis et al., 1991b; Constabel et al., 2000). However, transcripts of *PtdTI3* were also clearly induced below the wounded region, in leaves 18-20 and 27-29. Furthermore, *PtdTI3* transcripts were induced in roots, albeit at a low level. This experiment showed that, at least as seen for accumulation of *PtdTI3* mRNA, simulated herbivory elicits a defense response basipetal to the damaged region, and confirms our previous findings with MJ treatment (Fig. 4-1).

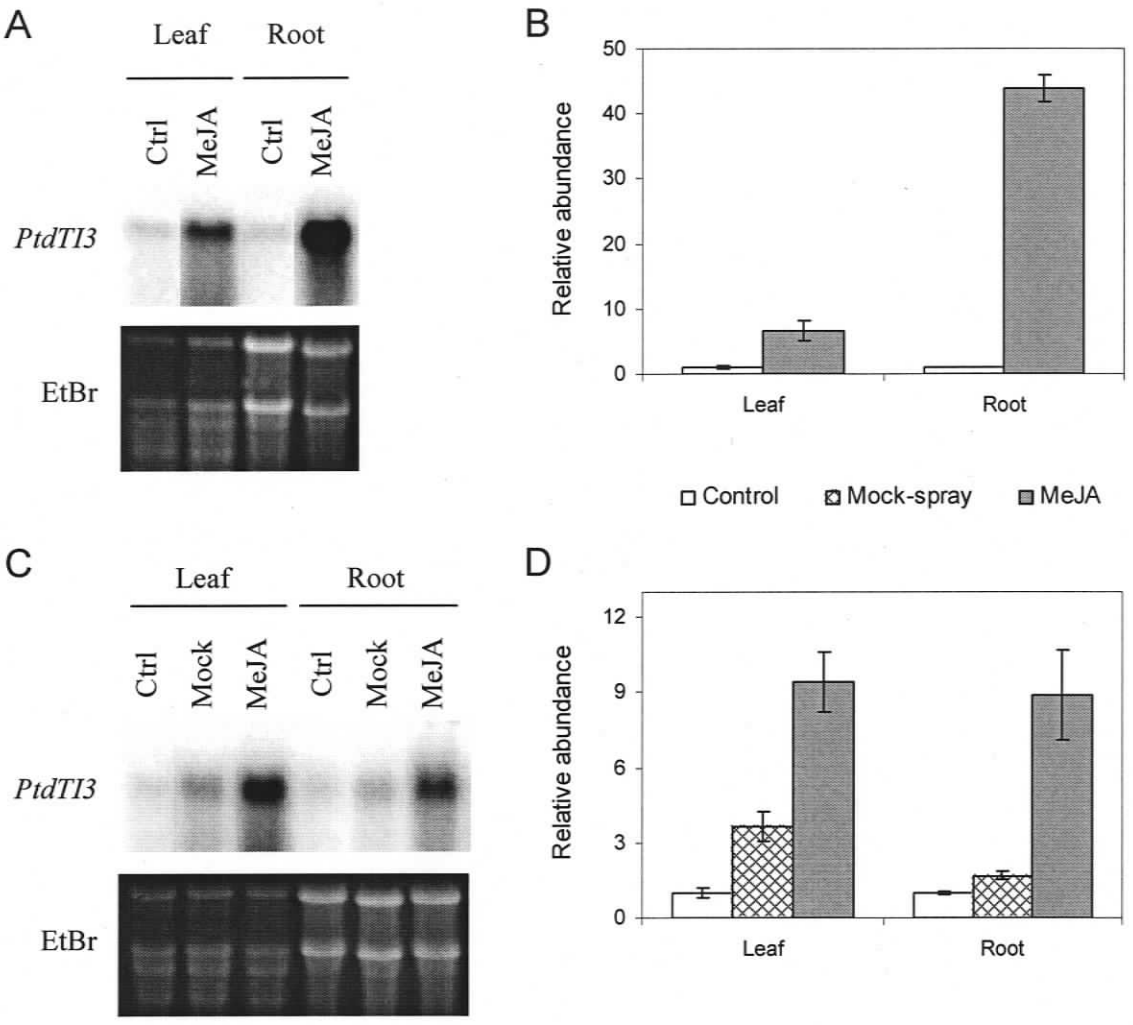


Figure 4-1. Accumulation of *PtdTI3* mRNA in leaves of plants treated with methyl-jasmonate (MJ).

MJ was applied to roots by irrigation (**A**, **B**) or shoots by spraying leaves (**C**, **D**). Leaves of LPI 9 – 11 and mature root were harvested 24-h after start of MJ treatment and analyzed by northern blot analysis. Experiments were replicated four times for MJ applied to roots and three times for MJ applied to shoots. Representative northern blots are shown (**A**, **C**). Transcript abundance from northern blot was quantified and normalized to levels of 25S rRNA from ethidium bromide staining (**B**, **D**). Open bars are untreated control plants, hatched bars are mock-treated control plants (**B**, **D** only) and grey bars are MJ-treated trees. Error bars are standard error.

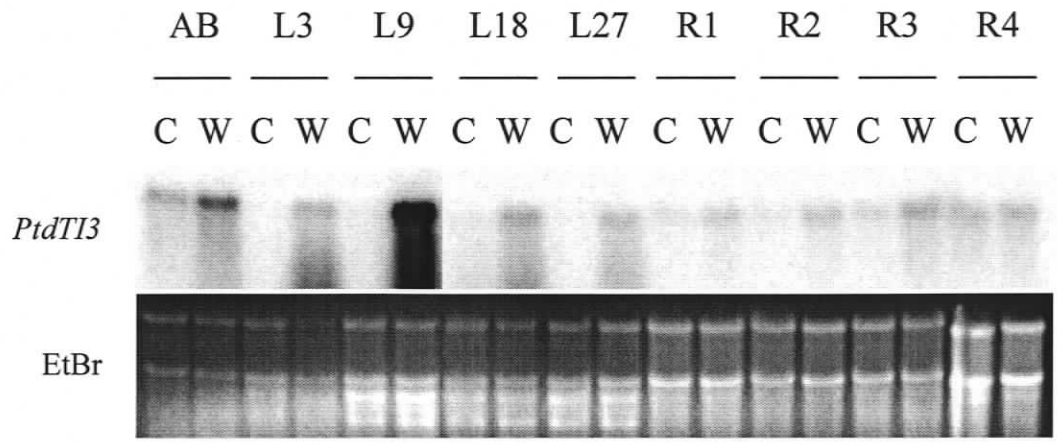


Figure 4-2. Accumulation of *PtdTI3* mRNA in tissues of plants wounded with pliers.

Leaves of LPI 9-17 were wounded, and 24 h after start of treatment tissues were harvested and analyzed by northern analysis. AB, apical bud; L3, leaves LPI 3-5; L9, leaves LPI 9-11; L18, leaves LPI 18-20; L27, leaves LPI 27-29; R1, main root (root < 10 cm from surface); R2, roots < 5 cm from main root; R3, root 10-20 cm from main root; R4, peripheral roots (root < 10 cm from root tip). Ethidium bromide staining is shown as a loading control.

Moreover, it suggested that mechanical wounding of leaves, a useful proxy for herbivory in poplar, is capable of inducing a defense response in poplar roots.

Wounding of leaves induces levels of PtdTI3 mRNA, protein and trypsin inhibitor activity in roots

To confirm that a defense response is induced in roots of wounded plants (Fig. 4-2), we conducted additional wounding experiments and used additional techniques to analyze the defense response in roots. To measure transcript levels, we replicated the experiment from Fig. 4-2 multiple times and quantified transcripts of *PtdTI3* in leaves and roots (Fig. 4-3A). We measured an average fold change of *PtdTI3* mRNA of 7.4X in plier-damaged leaves, comparable to our previous results (Chapter 2), and a fold change of 2.7X in roots. We next analyzed levels of TI3 protein; for this analysis, tissues were harvested later (4 days after foliar wounding) and TI3 protein levels in roots were assessed by western blot analysis. In six replicate experiments, TI3 protein was consistently induced in roots, with an average induction level of approx. twofold (1.76X; Fig. 4-3B). We further measured trypsin inhibitor activity of root extracts from control and leaf-wounded saplings of a representative experiment. Using linear regression analysis, we found that trypsin inhibitor activity was 1.34X higher in roots of leaf wounded saplings than control saplings (Fig. 4-3C), and although small, this difference was significant ($P = 0.002$). Together, these data demonstrate that the induced defense response in roots is not restricted to changes in transcript abundance, but that the response is also translated into increased protein accumulation and inhibitory activity.

Expression profiling reveals similarities in responses induced in roots and shoots

To study the transcriptional responses of poplar roots to wound and MJ treatment of leaves further, we conducted an analysis using cDNA macroarrays constructed previously (Chapter 2). We treated leaves of saplings with MJ or plier wounding, and separately analyzed the response in leaves and mature roots. Gene expression ratios were determined from comparison of three independent control and treated replicate plants (six plants total). Genes were considered to be differentially expressed on the basis of a $\geq$

Figure 4-3. Induction of trypsin inhibitor in roots of plier-wounded trees.

Leaves of LPI 9-17 (**A**) or all unfolded leaves (LPI ≥ 1)(**B**, **C**) were wounded with pliers, and leaves of LPI 9 - 11 and mature root were harvested 24-h (**A**) or 4-days (**B**, **C**) after start of treatment. Samples were analyzed by northern blot analysis (**A**), western blot analysis (**B**) or a trypsin inhibitor activity assay (**C**).

A. Accumulation of *PtdTI3* (Kunitz trypsin inhibitor 3) mRNA was quantified from northern blot and normalized to levels of 28S rRNA from ethidium bromide staining. Open bars are untreated control trees and grey bars are wounded trees. The experiment was replicated eight times, and error bars indicate standard error.

B. Western blot of wound-induced accumulation of TI3 protein. Two distinct experiments are shown.

C. Inhibitory activity of poplar root extracts against trypsin. Increasing amounts of crude extract from unwounded control (×) and wounded saplings (♦) were analyzed with a constant amount of trypsin. Linear regression analysis revealed that the difference in inhibitory activity between control and wounded extracts is significant ($P = 0.002$). Errors bars show standard error.

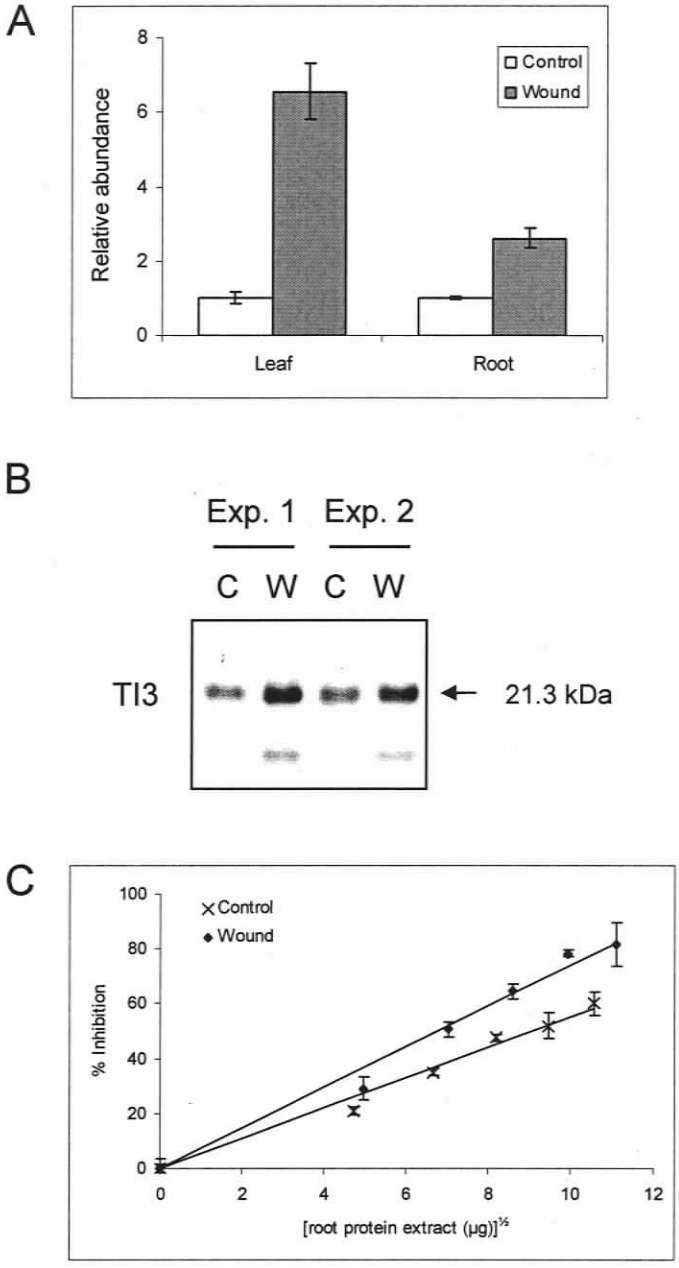

A
Relative abundance
8
6
4
2
0
Control
Wound
Leaf
Root
B
Exp. 1
Exp. 2
C W C W
TI3
21.3 kDa
C
% Inhibition
100
80
60
40
20
0
Control
Wound
0
2
4
6
8
10
12
[root protein extract (µg)]½

Figure 4-3.

twofold change in expression (either up- or down-regulation) and a significance of $P < 0.05$ as determined by a Student's *t*-test for the three replicates (Chapter 2). We previously used the same arrays and protocols to study wound and caterpillar-regurgitant induced responses in leaves and in that study, we rigorously tested and validated the array data using northern analysis. Since our macroarrays contained cDNAs derived from leaves, we first inspected the transcript abundance of this gene set in root samples and found that, as expected, many had extremely low signals on the arrays. We excluded these genes from the analysis, which ensured that we only examined genes that are truly expressed in roots.

Analysis of the induced gene set indicated that MJ and wounding leaf treatments significantly up-regulated 29 and 20 genes, respectively, in roots. We also found genes that were down-regulated, though they were fewer in number; MJ and wounding significantly down-regulated 2 and 3 genes, respectively, in roots. The leaf response was analyzed in detail previously; 163 and 108 genes were induced in leaves by MJ and wounding, respectively (Chapter 2 and 3). We compared our macroarray data from the four treatments (MJ leaves, wound leaves, MJ roots, wound roots) using Venn diagrams to determine how similar the inducible defense responses were between leaves and roots for our gene set (Fig. 4-4). In roots, MJ and wounding treatment of leaves appeared to induce overlapping responses, as 19 of 20 wound-inducible genes were also induced by MJ. Most root-induced genes were also induced in leaves (19 of 20 by wounding treatment; 19 of 29 by MJ treatment), but clearly the array contains many more genes inducible in leaves than in roots.

Inspection of the non-redundant genes induced in roots and leaves further highlights similarities between the leaf and root responses. The most strongly root-induced genes on the array encode known leaf defense-related poplar genes, including trypsin inhibitors, endochitinases, and vegetative storage protein (VSP) *win4.5* (Table 4-1, upper portion of top section). Furthermore, several genes that we previously reported as strongly induced in leaves were also up-regulated in the roots, including apyrase, cinnamyl alcohol dehydrogenase, acid phosphatase, CN192936 and CN193314 (proteins of unknown function), and cytochromes P450 (Chapter 2). In addition, we found four genes that were induced in roots but not leaves (Table 4-1, lower section), although these

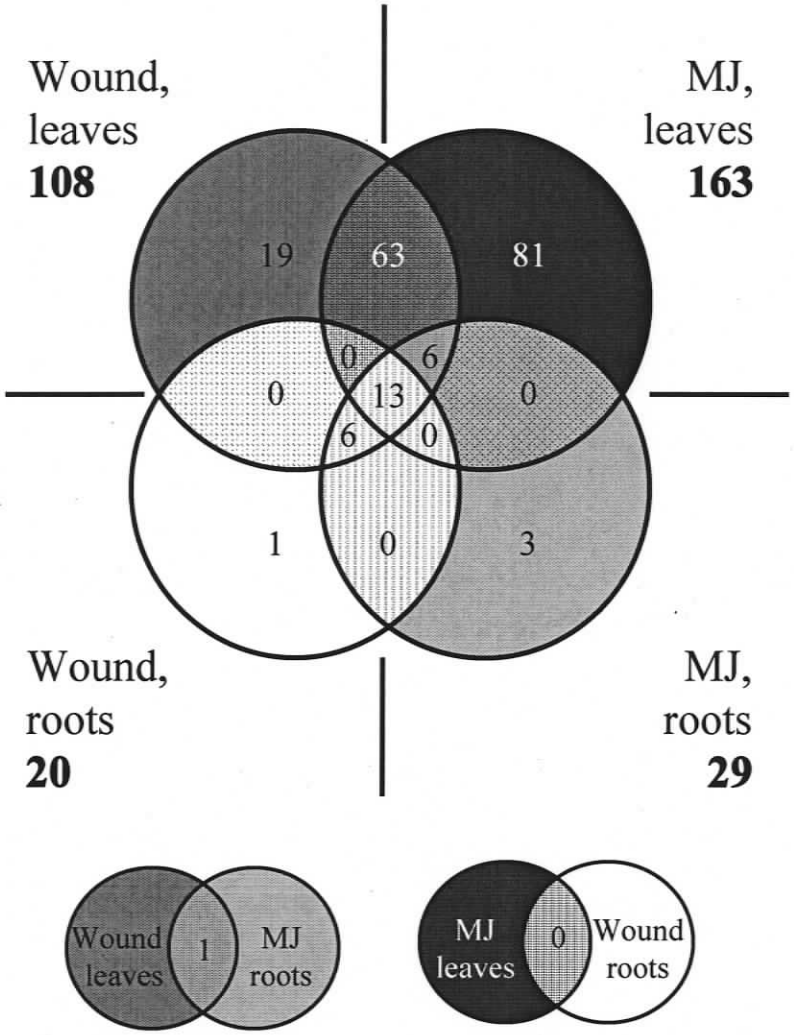


Figure 4-4. Comparison of gene expression after wound or MJ treatment in leaves and roots as determined by macroarray analyses.

Venn diagrams represent distribution of transcripts significantly induced (> two-fold induction, $P < 0.05$) by plier wounding or MJ treatment in leaves or roots.

Table 4-1. Macroarray data showing the effect of leaf wounding or foliar MJ treatment on gene expression in roots.

Putative function	GenBank accession	JGI ID[a]	AGI accession[b]	E-value	Wound treatment		MJ treatment	
					FC ± SE[c]	P^d	FC ± SE[c]	P^d
Genes significantly up-regulated in both roots and leaves								
Endochitinase *win8*	CN192595	694264	At3g12500	e-87	2.61 ± 0.50	0.018	21.35 ± 2.47	<0.001
Endochitinase *win6.2C*	CN192741	649163	At3g12500	e-100	4.09 ± 0.58	0.005	16.07 ± 0.50	<0.001
Apyrase	CN193208	573883	At5g18280	e-116	9.34 ± 3.41	0.029	10.38 ± 5.01	0.040
Kunitz trypsin inhibitor *PtdTI5*	CN192805	574326	At1g73325	e-09	6.84 ± 1.45	0.008	7.48 ± 2.48	0.012
Kunitz trypsin inhibitor *PtdTI3*	CN192549	739064	At1g73325	e-09	4.36 ± 1.36	0.022	8.69 ± 2.49	0.010
Kunitz trypsin inhibitor *PtdTI4*	CN193330	555576	At1g17860	e-28	3.68 ± 0.81	0.020	6.47 ± 1.70	0.015
Unknown protein	CN192936	596936	At3g03150	e-26	3.50 ± 0.31	0.003	5.34 ± 0.87	0.005
Vegetative storage protein *win4.5*	CN192930	571436	At4g24340	e-49	3.50 ± 0.26	0.002	5.13 ± 0.83	0.006
Cinnamyl alcohol dehydrogenase	CN192800	684659	At4g39330	e-112	2.88 ± 0.78	0.046	3.94 ± 1.53	0.038
Lil3 protein	CN193322	702880	At4g17600	e-72	1.98 ± 0.19	0.010	4.66 ± 0.43	0.002
Acid phosphatase, class B	xxxxxxxx	705837	At4g25150	e-67	2.80 ± 0.57	0.018	3.79 ± 0.36	0.003
Cytochrome P450	CN193273	589289	At5g07990	e-100	3.28 ± 0.87	0.025	2.86 ± 0.08	<0.001
Poplar *Pop3 / SP1*	xxxxxxxx	687234	At3g17210	e-21	1.86 ± 0.27	0.028	3.88 ± 0.15	<0.001
Cytochrome P450	CN193412	645826	At5g07990	e-102	2.73 ± 0.71	0.039	2.97 ± 0.12	<0.001
Unknown protein, ZIM motif	CN193314	645806	At1g19180	e-31	2.17 ± 0.36	0.027	3.52 ± 0.55	0.008
Cytochrome P450	CN193236	553609	At5g07990	e-104	2.39 ± 0.76	0.114	2.78 ± 0.90	0.045
Cytochrome P450	CN193274	645827	At5g07990	e-40	2.28 ± 0.49	0.045	2.78 ± 0.43	0.010
Hydroperoxide lyase	CN192806	688325	At4g15440	e-139	1.95 ± 0.19	0.012	2.61 ± 0.35	0.009
18S rRNA gene	CN192944	670269	At3g41768	e-56	1.73 ± 0.28	0.050	2.37 ± 0.53	0.030
Genes significantly up-regulated in roots only								
ATP synthase, gamma chain	CN192816	555320	At4g04640	e-153	1.92 ± 0.11	0.004	3.57 ± 0.99	0.037
Zinc finger (B-box type) protein	CN193382	690491	At5g57660	e-79	2.07 ± 0.53	0.060	2.92 ± 0.87	0.035
Galactinol synthase	CN192679	565191	At1g60470	e-156	1.06 ± 0.16	0.417	3.57 ± 0.40	0.004
O-Methyltransferase	CN193109	582793	At4g35160	e-15	2.60 ± 0.49	0.021	1.99 ± 0.46	0.077

[a]JGI protein ID of the JGI gene model from the *P. trichocarpa* genome (http://genome.jgi-psf.org/Poptr1_1/Poptr1_1.home.html) that corresponds to the EST.

[b]AGI (Arabidopsis Genome Initiative) code with expect value for best match of poplar gene (JGI gene model) to *Arabidopsis thaliana* determined by BLASTX of TAIR6 (http://www.arabidopsis.org/). For 18S rRNA gene (CN192944), the best match was determined by BLASTN.

[c]Mean fold change (± SE) of gene expression in roots of wound- and MJ-treated plants. Shading is proportional to fold change to facilitate visual comparisons.

[d]Significance of treated plants compared to control plants, as determined by a Student's *t*-test.

genes showed only modest transcript increases. Our *in silico* analysis of public EST databases confirmed that these four genes are expressed in roots (Table 4-2) and they therefore indicate potential root-specific defense responses.

Some genes strongly induced in leaves are not expressed in roots

We found that many genes that are strongly induced in leaves were not at all up-regulated in the roots or were expressed below our threshold for reliable expression. This list includes polyphenol oxidase *PtdPPO1* (GenBank accession CN193334; JGI protein ID 568791), class 3 lipase (CN192786; 713858), VSP *pni288* (CN193425; 573862), 13-lipoxygenase (GenBank CN192531), acyl-activating enzyme (CN192663; 556023), β-amylase (CN192760; 679498) and *PtdPop3-/SP1-like* (JGI protein ID 723971), all of which we have previously shown to be among the most strongly induced genes in leaves (Chapter 2). The inclusion of polyphenol oxidase and class 3 lipase on this list is especially interesting, since these genes have the highest inductions in leaves, but were only marginally up-regulated in roots, and only after foliar MJ treatment. Our data therefore suggest that some defense genes are specifically expressed in leaves, while others are induced in both leaves and roots. Thus, the macroarray analysis illustrates both similarities and differences among root and leaf responses, although we emphasize that this is only a partial picture due to the limited scope of our array.

Defense protein levels in roots of leaf wounded saplings correlate with gene expression

We used western blot analysis to provide some validation of our results, and to test if the transcriptional changes in roots also translate into increases in the corresponding proteins. We used available polyclonal antibodies raised against VSP WIN4, PPO1 and Pop3-/SP1-like to detect changes in protein levels in roots 4 d after leaf wounding. Our macroarray analysis indicated that, like *PtdTI3*, *VSP win4.5* was up-regulated in roots, while *PtdPPO1* and *PtdPop3-/SP1-like* exhibited no change in expression (Table 4-1 and data not shown). Our western blot indicated that VSP WIN4 protein levels increased after leaf wounding in multiple experiments (Fig. 4-5), with an average protein induction of 1.7 X, which compared well with the 3.50X increase in

Table 4-2. *In silico* validation of root expression for genes marked as induced in roots[a].

Putative identity	GenBank accession	JGI ID[b]	No. ESTs[c]
Poplar endochitinase *win8*	CN192595	694264	5
Poplar endochitinase *win6.2C*	CN192741	649163	1
Apyrase	CN193208	573883	0
Kunitz trypsin inhibitor *PtdTI5*	CN192805	574326	1
Kunitz trypsin inhibitor *PtdTI3*	CN192549	739064	28
Kunitz trypsin inhibitor *PtdTI4*	CN193330	555576	1
Unknown protein	CN192936	596936	3
Poplar vegetative storage protein *win4.5*	CN192930	571436	2
Cinnamyl alcohol dehydrogenase	CN192800	684659	4
Lil3 protein	CN193322	702880	0
Acid phosphatase, class B	xxxxxxxx	705837	8
Cytochrome P450	CN193273	589289	0
Poplar *Pop3 / SP1*	xxxxxxxx	687234	3
Cytochrome P450	CN193412	645826	2
Unknown protein, ZIM motif	CN193314	645806	3
ATP synthase, gamma chain	CN192816	555320	1
Cytochrome P450	CN193236	553609	0
Cytochrome P450	CN193274	645827	1
Zinc finger (B-box type) protein	CN193382	690491	2
Galactinol synthase	CN192679	565191	1
O-Methyltransferase	CN193109	582793	2
Hydroperoxide lyase	CN192806	688325	0

[a]PopulusDB (http://poppel.fysbot.umu.se/ Sterky et al., 2004), PoplarDB (http://mycor.nancy.inra.fr/PoplarDB/index.html Kohler et al., 2003), and The Gene Index Databases (Quackenbush et al., 2001) were queried using BLASTN with the corresponding JGI gene model to measure *in silico* gene expression in roots.
[b]JGI protein ID of the JGI gene model from the *P. trichocarpa* genome (http://genome.jgi-psf.org/Poptr1_1/Poptr1_1.home.html) that corresponds to the EST.
[c]Total number of ESTs identified from PopulusDB, PoplarDB and the Poplar Gene Index. For all BLASTN queries, the highest smallest sum probability or E-value was 5e-67; the lowest was 0.

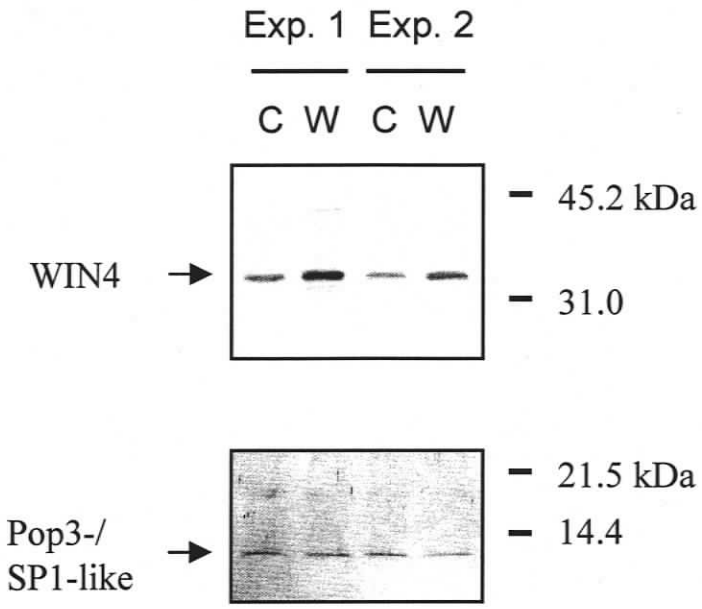


Figure 4-5. Accumulation WIN4 and Pop3-like protein in roots of plier-wounded trees from two distinct experiments.

Leaves of the entire tree were wounded with pliers and mature roots were harvested 4-days after start of treatment and analyzed by western blot analysis.

transcript levels by macroarray analysis. By contrast, the Pop3-/SP1-like protein was present in roots, but its levels did not increase after leaf wounding (Fig. 4-5). The PPO1 protein isoform was not expressed in roots (data not shown), again consistent with *PtdPPO1* mRNA levels which were below the threshold for reliable expression on the macroarray. Therefore, overall these defense protein levels all correspond well with the pattern of transcripts detected by macroarray analysis.

4.4 Discussion

Our results clearly demonstrate that a defense response is induced in poplar roots by simulating herbivory of leaves. Treatment of poplar sapling shoots with MJ or mechanical wounding with pliers resulted in bidirectional systemic signaling, including the systemic induction of defense genes in roots. The response in roots after foliar wounding was observable at mRNA, protein and activity levels. Comparative macroarray analyses indicated some similarity between the responses of leaves and roots after foliar wounding and MJ treatments, as many genes induced in roots are poplar defense genes first described from leaves. However, some genes strongly induced in leaves were not expressed in roots. To our knowledge, this is the first application of transcript profiling to study a systemic defense response in roots after leaf damage.

Our findings indicate that a systemic wound signal moves downward from the shoot into the root. Since JA is known to induce defense in an acropetal fashion in tomato and other plants it is also likely that JA or a JA derivative is transported from leaves to roots and is therefore responsible for the basipetal systemic defense response. In tobacco, radiolabeled JA applied to leaves has been shown to be translocated to roots (Zhang and Baldwin, 1997). In general, the movement of the systemic signal is governed by vasculature and source-sink relations (Davis et al., 1991b; Jones et al., 1993; Orians et al., 2000; Schittko and Baldwin, 2003). Interestingly, a recent study of *Nicotiana attenuata* showed that simulated foliar herbivory elicits rapid changes in source-sink relations, increasing the transport of photoassimilate to roots (Schwachtje et al., 2006). A similar response has also been observed for *Populus*, as foliar treatment with jasmonic acid increases carbon allocation to roots (Babst et al., 2005). Increased root sink strength would thus predict that a systemic signal moves basipetally into the root, although in this

situation it is unclear if the response in roots is an artifact of source-sink shifts or a defense response *per se*. Source-sink relations likely explain why we observed stronger acropetal than basipetal systemic responses, as younger tissues above the wounded region are stronger sinks than the mature leaves and roots. Nevertheless, our data provide strong evidence for bi-directional systemic signaling in poplar. This was previously shown for potato, tobacco and poplar (Pena-Cortes et al., 1988; Jones et al., 1993; Schittko and Baldwin, 2003), although in poplar Davis et al. (1991b) did not observe basipetal induction of *win3*, a distinct Kunitz trypsin inhibitor. This discrepancy is likely an artifact of their choice of marker gene, since wound-induced accumulation of *win3* transcript occurs primarily in young leaves (Patton and Constabel, unpublished data). By contrast, our marker for the inducible systemic response, *PtdTI3*, is inducible in both young and mature tissues.

Why a defense response is induced in roots of poplar after foliar wounding is not clear. The induced genes may constitute a general defensive response, and induction of defense genes in roots is likely to provide some enhanced resistance against belowground herbivores. Several genes we found to be up-regulated in roots are predicted to have negative effects on root pests. For example, the two most strongly induced genes in roots are endochitinases, one of which was recently shown to directly inhibit insect development (Lawrence and Novak, 2006). In roots of *Citrus* species that are attacked by citrus root weevil (*Diaprepes abbreviatus*), endochitinase is an induced defense and root extracts degrade the peritrophic matrix of weevil larvae (Mayer et al., 1995). Our experiments also identified three KTIs as strongly induced in roots. In tomato, root-knot nematode (*Meloidogyne javanica*) infection and root wounding increase transcript and protein levels of a KTI in roots (Brenner et al., 1998). In sugar beet, resistance against beet cyst-nematodes (*Heterodera schachtii*) is enhanced by transgenic overexpression of a KTI (Cai et al., 2003), and thus it is likely that KTIs enhance resistance against belowground herbivores. In poplar, *PtdTI3* in particular is among the most strongly induced genes in leaves, and is expressed in almost all plant tissues. Recombinant TI3 protein inhibits a range of proteases and is an effective inhibitor of larval midgut proteases of FTC and bertha armyworm (*Mamestra configurata*) (Major and Constabel, unpublished data). These root-induced poplar defense genes are therefore likely to

participate in resistance to belowground herbivory and provide tantalizing clues for future experiments.

Compared to the acropetal induction of *PtdTI3* mRNA after leaf wounding, we observed only a modest basipetal induction. While MJ elicited a stronger basipetal induction, we cannot exclude that the exogenous MJ was translocated to roots (Zhang and Baldwin, 1997) rather than triggering a systemic defense response. In addition, the basipetal systemic defense response may be stronger at other timepoints, and our analyses were only carried out at a single timepoint (24 h for analysis of transcript levels and 4 d for protein levels and activity). Genes with transient or delayed expression patterns have been shown for studies of inducible root defense (Alkharouf et al., 2006), and would have escaped detection by our array analysis. Furthermore, the composition of our arrays limited our analysis, since they were constructed with ESTs generated from a leaf cDNA library. This was reflected by the small number of genes up-regulated in roots compared to leaves (Fig. 4-4). To improve the confidence of our array expression results, we performed an *in silico* validation of the array results using publicly available root EST databases to independently confirm the expression of genes marked as root-expressed on the array. Among the 23 genes that were induced in roots, only five were not represented at least once by a root-derived EST (Table 4-2). Thus the majority of array elements identified by our analysis do correspond to genes expressed in roots.

While defense responses in roots have not received the same attention as responses in shoots, herbivore-mediated interactions between belowground and aboveground plant organs are known (reviewed by Blossey and Hunt-Joshi, 2003; van Dam et al., 2003). However, few studies have investigated these inducible responses from a molecular perspective. We have used a molecular approach to describe basipetal systemic defense signaling in poplar. Poplar roots not only generate a systemic signal, but they also respond to systemic signals generated by simulated herbivory of leaves. Moreover, we have shown that this defense response in roots is manifested at the mRNA and protein levels. Our use of transcript profiling has provided preliminary evidence that the transcriptional changes that occur as part of a root defense response has significant overlap with the leaf defense response. The use of whole genome arrays or arrays with root-derived ESTs should provide a better understanding of the induced root response.

5 Biochemical characterization and functional diversity of the Kunitz protease inhibitor gene family of poplar

[The following chapter is in preparation for submission to Plant Physiology]

5.1 Introduction:

Plants respond to challenge by insect herbivores with an active induction of defenses which can include both proteins and secondary metabolites that are toxic, antifeedant or antinutritive. For example, in many plants herbivory triggers active synthesis of antinutritive proteinase inhibitor proteins (PIs), which inhibit insect digestive proteases and lead to lowered growth rates or starvation (Ryan, 1990). Induced antinutritive proteins also include the oxidative enzymes polyphenol oxidase (PPO), peroxidase and lipoxygenase, thought to act by destroying or modifying essential amino acids and fatty acids (Duffey and Felton, 1991), and the amino acid-degrading enzymes arginase and threonine deaminase, which deplete levels of the essential amino acids arginine and threonine, respectively, in midguts and reduce insect growth (Chen et al., 2005; Chen et al., 2007). Induced plant cysteine proteases are toxic proteins that permeabilize the peritrophic matrix of insect herbivores and reduce insect growth (Pechan et al., 2002; Konno et al., 2004; Mohan et al., 2006). In some species, the synthesis of secondary metabolites such as alkaloids, terpenoids, glucosinolates and phenolics is upregulated in response to herbivory and enhances insect resistance (Walling, 2000; Kessler and Baldwin, 2002).

Plant PIs consist of at least ten nonhomologous families recognized by their amino acid sequence and which inhibit each of the four mechanistic classes of proteinases (Laskowski and Kato, 1980; De Leo et al., 2002; Rawlings et al., 2004). Most plant PIs inhibit serine proteinases, such as the well-characterized Kunitz and Bowman-Birk PI families. The Kunitz-type PIs, referred to here as Kunitz trypsin inhibitors (KTIs), are proteins of approx. 20 kDa with one or two disulfide bonds and a single reactive site; by comparison, the Bowman-Birk PIs are smaller (approx. 8 - 10 kDa) with high cysteine content and two reactive sites (Richardson, 1991). The interaction of KTIs with their cognate protease is well-characterized; the reactive site of the KTI binds tightly to the

protease active site in a substrate-like manner. Hydrolysis is extremely slow and at equilibrium the KTI exists between free enzyme and complexed with the protease (Laskowski and Kato, 1980). The KTI is effectively bound to the protease and acts as a competitive inhibitor. Although the reaction is reversible, some KTIs have such strong affinities for their protease that the reaction can be considered irreversible (Beynon and Bond, 1989). The structure of plant KTIs is a β-trefoil fold that consists of 10-12 antiparallel β-strands connected by long loops (Song and Suh, 1998). The reactive site is located on a protruding reactive loop, which forms H-bonds with the active groove of the substrate protease. The reactive loop is stabilized by extensive intramolecular H-bonding and disulfide bridges, allowing the loop to remain tightly bound to the protease even after hydrolysis. Most KTIs have four conserved cysteine residues that form two disulfide bridges, although some KTIs have been found with only one disulfide bridge, or no disulfide bridges. While evidence from several studies suggests that at least the first disulfide bridge, which surrounds the reactive loop, is necessary for inhibitor activity (DiBella and Liener, 1969; do Socorro et al., 2002), a few KTIs do not appear to require any disulfide bonds (Araujo et al., 2005).

Plant KTIs have extremely diverse targets and thus can have negative effects on a broad range of phytophagous pests and pathogens. While many plant KTIs inhibit trypsin or chymotrypsin, some inhibit other serine proteinases such as elastase (Valueva et al., 2000; Sumikawa et al., 2006) and subtilisin (Terada et al., 1994; Revina et al., 2004), cysteine proteinases (Rowan et al., 1990), and aspartic proteinases such as cathepsin D (Ritonja et al., 1990) and aspergillopepsin (Heibges et al., 2003b). Moreover, not all Kunitz-like proteins are protease inhibitors, including proteins with lectin-like carbohydrate binding activity (Macedo et al., 2004) and invertase inhibitor activity (Glaczinski et al., 2002). Some plant KTIs are also effective antimicrobial proteins, presumably via inhibition of microbial proteinases (Macedo et al., 2004; Kim et al., 2005; Park et al., 2005). It is thus especially important to demonstrate the biological function of plant KTIs, considering their variable functions.

Although the activity of many plant KTIs are known, only in potato tubers has a gene family been characterized. The potato genome encodes a multigene family of at least 21 KTIs that can be classified into three major homology groups (Ishikawa et al.,

1994; Heibges et al., 2003a). Analysis of KTI sequences and inhibitor functions demonstrates the diversity of this family, including nonsynonomous and indel polymorphisms that translate into functional diversity both within and across the homology groups (Heibges et al., 2003a; Heibges et al., 2003b).

In *Populus*, analysis of the wound-inducible KTI *win3* from hybrid poplar was first described by Gordon and co-workers (Bradshaw et al., 1990; Hollick and Gordon, 1993, 1995). We later isolated wound-inducible *P. tremuloides TI1* and *TI2* genes, two KTI paralogs from a recent duplication of *win3*. We also described wound-inducible accumulation of their transcripts and proteins, as well as *in vitro* inhibitor activity, which is consistent with a role for these KTIs in herbivore defense (Haruta et al., 2001a). More recently, new wound-inducible KTIs from hybrid poplar have also been identified via expressed sequence tag (EST) gene discovery programs (Christopher et al., 2004; Ralph et al., 2006a), and transcript profiling studies have shown them to be among the most strongly induced genes after wounding and herbivory (Chapter 2; Ralph et al., 2006a). The *P. trichocarpa* genome is now fully sequenced (Brunner et al., 2004; Tuskan et al., 2004), and large EST collections are available and can be used for digital analysis of gene expression and gene discovery (Sterky et al., 2004).

Here we use the poplar genome to identify the entire poplar KTI gene family, and characterize the inhibitor activities of five representative members expressed heterologously as recombinant proteins in *E. coli*. These KTI genes are wound-inducible, and therefore likely part of the herbivore defense response. We find that the poplar KTI genes tested encode functional inhibitors but have distinct inhibitor profiles and substrate preferences. We also show that these proteins are biochemically distinct. Furthermore, the combined inhibitor activity and expression patterns of the KTIs correlate with proteinase inhibitor activity of poplar leaves. Finally, we show that at least one of the KTIs is an active inhibitor of larval midgut proteases from *Mamestra configurata* demonstrating that at least one of these KTIs is an active inhibitor of insect proteases, consistent with the probable role of this family as an inducible defense of *Populus* species.

5.2 Materials and Methods:

Plant material

Poplar hybrid H11-11 (*Populus trichocarpa* × *P. deltoides*), originating from the University of Washington/Washington State University Poplar Research Program, were propagated from greenwood cuttings in Sunshine Mix #4 (Sungro, Seba Beach, AB, Canada) in 0.25 L propagation containers (RootMaker, Huntsville, AL, USA). After plantlets had rooted and reached a height of approximately 10 cm, they were transplanted to 15 cm-diameter pots containing Sunshine Mix #4 plus slow-release nutrients (8.9 g L^{-1} controlled release 8-6-12 NPK plus micronutrients (Acer, Delta, BC, Canada), 0.458 g L^{-1} superphosphate 0–20–0 (Green Valley, Surrey, BC, Canada), 1.21 g L^{-1} Micromax Micronutrients (Scotts-Sierra, Marysville, OH, USA), and 4.75 g L^{-1} Dolomite lime (IMASCO, Surrey, BC, Canada)). Plants were maintained in the Bev Glover Greenhouse at the University of Victoria. Supplemental lighting from 600 W high pressure sodium lamps was used to extend the photoperiod to 16/8 hr, and the temperature within the greenhouse was maintained at 25/18°C. Plants were watered daily with a solution containing 0.1 g L^{-1} 20-20-20 PlantProd fertilizer (Plant Products, Brampton, ON, Canada). All lateral shoots were pruned as they developed so that each plant consisted of a single main stem, no less than 2 weeks prior to experiments.

Wounding treatments

Plants were 12 weeks old and 1 m tall with approximately 30 leaves when used for wounding experiments. Leaves were numbered using the Leaf Plastochron Index (Larson and Isebrands, 1971), with the index leaf (LPI 0) defined as the first developing leaf with a lamina length of 20 mm. Leaf margins of all unfolded leaves (LPI > 0) were wounded by crushing with pliers, three times, at 1 h-intervals. This wounding method elicits a strong defense response including accumulation of KTI proteins (Haruta et al., 2001a). Tissue was collected at indicated times after start of treatment. Immediately after harvesting, tissues were frozen in liquid nitrogen, and stored at -80°C until analyzed.

Protein extraction and western blot detection

Protein was extracted with Na_2HPO_4 buffer (100 mM, pH 7.0) containing 0.1% Triton X-100, 5% w/v polyvinylpolypyrrolidone, and 1% 2-mercaptoethanol. Extracts were clarified by centrifugation, and soluble protein quantified (Bradford, 1976). For western blotting, proteins were separated by SDS-PAGE and electro-transferred onto PVDF membranes (Pierce, Fisher Canada, Nepean, ON, Canada), and Ponceau S (Sigma, St. Louis, MO, USA) staining was used to verify equal loading and transfer efficiency. Western blot detection was carried out using polyclonal antibodies raised against TI2 (Haruta et al., 2001a) and TI3 (see below). Immunocomplexes were detected using acid phosphatase- or horseradish peroxidase-conjugated secondary antibodies (Bio-Rad, Hercules, CA, USA) and blots were developed colourimetrically with reagents 5-bromo-4-chloro-3-indoyl phosphate (BCIP, Pierce) and nitroblue tetrazolium chloride (NBT, Pierce; acid phosphatase) or 3,3'-diaminobenzidine tetrahydrochloride (DAB, Sigma; horseradish peroxidase).

Identification of KTIs from poplar genome, phylogenetic analysis and digital northerns

To obtain the complete poplar KTI family, the *Populus trichocarpa* genome sequence (http://genome.jgi-psf.org/Poptr1/Poptr1.home.html) was queried for gene models annotated as Kunitz-type PIs. These models were combined with previously identified *Populus* KTI sequences (Bradshaw et al., 1990; Hollick and Gordon, 1993; Haruta et al., 2001a; Christopher et al., 2004) and used to query the genome again for any Kunitz-like proteins not annotated as such. Gene models were removed that were not KTIs (i.e. have the Kunitz motif and at least one disulfide bond). Truncated gene models were used to query the GenBank plant EST database to try to identify full length sequences; truncated models without full length ESTs in GenBank were removed. Many of the gene models encoding truncated KTIs were flanked by regions of highly repetitive DNA and could not be resolved as separate TI genes. In particular, *GWIN3*, the first poplar KTI identified (Bradshaw et al., 1990), was localized to a region densely populated by repetitive DNA and truncated TI gene models. As such, no gene model corresponding to *GWIN3* is present in the current poplar genome. This is consistent with studies of the gene organization of *win3*; the *win3* region is hypervariable, with the locus

shown to contain repeats and consist of several clustered KTI genes (Bradshaw et al., 1990; Hollick and Gordon, 1993). Additional analysis will be required to resolve the gene organization at this locus. The *gwin3* and *win3.12* KTI sequences were therefore used for phylogenetic analysis, even though no corresponding sequence was identified in the genome. KTI gene models with nucleotide sequences ≥ 99% identical were considered allelic, and only one gene model per locus was used for phylogenetic analysis.

Multiple amino acid alignments were made using Clustal W with default parameters, followed by manual adjustments in BioEdit (http://www.mbio.ncsu.edu/BioEdit/bioedit.html). To improve aligments, secondary structure predictions were made using Jpred (http://www.compbio.dundee.ac.uk/~www-jpred/) and tertiary structure predictions were made using SWISS-MODEL (http://swissmodel.expasy.org/), CPHmodels (http://www.cbs.dtu.dk/services/CPHmodels/) and ESyPred3D (http://www.fundp.ac.be/sciences/biologie/urbm/bioinfo/esypred/). Predicted secondary structure and tertiary structures were compared and used to help align variable sites and indels. A neighbor-joining phylogenetic tree was constructed in MEGA 3.1 (http://www.megasoftware.net/) using protein distance with pairwise deletion of gaps. Bootstrapping was used to test the robustness of the tree in MEGA 3.1.

To determine preliminary KTI expression patterns, we queried the PopulusDB EST database (http://poppel.fysbot.umu.se/) which contains >100,000 EST sequences from 19 different tissue libraries (Sterky et al., 2004). An expression profile was constructed by counting the number of ESTs, according to their libraries, of each cluster.

Heterologous expression and purification of recombinant KTI proteins

TI2 was expressed and purified as described previously (Haruta et al., 2001a). Signal peptides of each poplar KTI were predicted using SignalP (http://www.cbs.dtu.dk/services/SignalP/). The coding sequence of poplar KTIs, minus the signal sequence, were PCR-amplified from plasmid clones. TI3 (EST ID H1059; GenBank accession CN192549) was amplified with the sense primer TI-3s (5'-CGGGATCCGAAGCAGTGATCGATGCC-3'; *Bam*HI site underlined) and the antisense primer TI-3a (5'-CCCAAGCTTCATCATTTTATACTCG-3'; *Hin*dIII

underlined). TI4 (H828; CN193330) was amplified with the sense primer TI-4s (5'-CGGGATCCTATACTGAGCCGGTGCTTG-3'; *Bam*HI site underlined) and the antisense primer TI-4a (5'-CCCAAGCTTTATGGATGAACTTAAAGG-3'; *Hin*dIII underlined). TI5 (H1685; CN192805) was amplified with the sense primer TI-5s (5'-CGGGATCCTCAGGGAATCCAGTGCTTG-3'; *Bam*HI site underlined) and the antisense primer TI-5a (5'-CCCAAGCTTTACAACAGCTTTTAATCC-3'; *Hin*dIII underlined). TI6 (WS0133_E08; DT502517) was amplified with the sense primer TI-6s (5'-CGGGATCCAAAGATGCTGCAGCAGTGCT-3'; *Bam*HI site underlined) and the antisense primer TI-6a (5'-CCCAAGCTTTTCATCTGGTTCAAACATAA-3'; *Hin*dIII underlined).

PCR products were digested with *Bam*HI and *Hin*dIII and cloned into the respective restriction sites of the pET21a (Novagen, EMD Biosciences, San Diego, CA, USA) bacterial expression vector. The resulting pET-TI3, pET-TI4, pET-TI5 and pET-TI6 plasmids were sequenced to confirm that the coding sequence was in frame and contained no mutations, and the plasmids were moved into *E. coli* strain BL21(DE3)(Novagen) for expression. Recombinant proteins were produced and isolated from bacterial inclusion bodies as described previously (Haruta et al., 2001a). The recombinant proteins were purified to homogeneity on a nickel affinity resin (Ni-NTA Agarose, Qiagen) and eluted along a pH gradient under denaturing conditions (8 M urea) as per the manufacturer's protocol. To renature the recombinant KTIs, we initially used our previous protocol (Haruta et al., 2001a) but found that the proteins aggregated and precipitated in buffers of different ionic strength, concentration of reducing agent and pH (Table 5-1). We therefore optimized the re-folding conditions for the KTIs and found a re-folding buffer suitable for all the proteins, such that subsequent *in vitro* assays for PI activity would not be influenced by different buffers. The recombinant KTIs were dialyzed against 50 mM Tris-HCl buffer (pH 8.0) at 4°C for at least 24 h with at least six changes of buffer. For comparison with *in vitro* assays of PI activity, soybean KTI (STI; Sigma) dissolved in ddH_2O was also dialyzed against 50 mM Tris-HCl buffer (pH 8.0) so that buffers were comparable. Protein concentrations were determined spectrophotometrically (A_{280}) using molar extinction coefficients by Vector NTI Advance

9.0 (Invitrogen, Burlington, ON, Canada) or reported by supplier (STI; Sigma), and were verified on Coomassie-stained SDS-PAGE gels.

For production of TI3 antibody, we precipitated TI3 protein by dialysis against PBS (phosphate buffered saline) at 4°C, since we previously observed that TI3 precipitates in PBS during optimization of re-folding conditions. TI3 protein was recovered by centrifugation and dried, assessed for purity by Coomassie-stained SDS-PAGE, and used for antibody production. Rats were immunized with 100 μg TI3 dry protein in Freund's complete adjuvant (Sigma, St. Louis, MO, USA), with booster injections of 50 μg protein using standard procedures.

Proteinase inhibitor assays with synthetic substrates

All PI activity assays were done in triplicate and the results shown as the means of three replicate assays. The PI activities of recombinant poplar KTIs against trypsin (EC 3.4.21.4; Sigma), chymotrypsin (EC 3.4.21.1; Sigma) and elastase (EC 3.4.21.36; Sigma) were determined by pre-incubating increasing concentrations of each KTI with a standard quantity of proteinase (6.6×10^{-4} g/L final assay concentration) in the appropriate assay buffer. PI activity was determined as described (Worthington, 1988), by measuring residual proteinase activity as the rate of hydrolysis of the chromogenic substrates TAME (for trypsin, p-toluene-sulfonyl-L-arginine methyl ester; Sigma) by monitoring the change in ΔA_{247}/min, BTEE (for chymotrypsin, benzoyl-L-tyrosine ethyl ester; Sigma) by monitoring the change in ΔA_{256}/min, and SucAla3pNA (N-succinyl-Ala-Ala-Ala-p-nitroanilide; Sigma) by monitoring the change in ΔA_{410}/min. STI (Sigma) and bovine serum albumin (BSA; Sigma) were assayed under identical conditions as a positive PI control and a negative protein control, respectively. The KTI concentrations required to inhibit 50% of the proteinase activity were calculated from the linear portion of the plot of residual proteinase activity against KTI protein (μg).

PI activity of leaf extracts was determined against trypsin, chymotrypsin and elastase by titrating leaf protein extracts with each proteinase, and residual proteinase activity was measured as described above. Percent proteinase inhibition was plotted against the square root of leaf protein extract (mg/mL). To directly compare levels of inhibitory activity in leaves, we calculated the total protein concentration of leaf extract

which inhibits 50% of proteinase activity (IC_{50}). For statistical comparison of inhibitor activity in leaves of control and wounding treatments, we compared slopes (% inhibition/(protein extract)^{½}) from linear regression analysis and calculated a *P*-value (two-tailed) testing the null hypothesis that the slopes are identical (PI activity is equal from control and wound treatments).

Proteinase inhibitor assays with the non-specific substrate azocasein

PI activities against subtilisin A (EC 3.4.21.62; Sigma) and papain (EC 3.4.22.2; Sigma) were determined by pre-incubating increasing concentrations of each KTI with a standard quantity of proteinase in the appropriate assay buffer for 10 min (for subtilisin, 50 mM Tris-HCl pH 7.5 at 37°C; for papain, 50 mM $NaCH_3COO$ pH 6.0, 2 mM DTT at 23°C). After azocasein (Sigma) was added to a final concentration of 1%, the reaction was followed for 1 h and stopped by the addition of trichloroacetic acid. The A_{450} was measured and used to calculate residual proteinase activity.

PI activity of midgut proteases from bertha armyworm (*Mamestra configurata*) was determined by pre-incubating increasing concentrations of each KTI with a standard quantity of midgut extract (Hegedus et al., 2003) in two assay buffers of different pH (0.1 M Tris-HCl pH 8.0; 0.1 M glycine-NaOH pH 11.0), which correspond to two major peaks of midgut protease activity. After azocasein was added to 1% final concentration, the reaction was followed for 6 h and stopped by the addition of trichloroacetic acid. The A_{450} was measured and used to calculate residual protease activity. STI was assayed under identical conditions, as a positive PI control.

Biochemical stability assays

For stability assays, poplar KTIs were incubated at increasing temperature, with increasing concentration of reducing agent, or were stored long-term at 4°C, after which PI activity was determined for the preferred proteinase substrate and compared with the activity of the respective untreated KTIs. After treatments, residual PI activity was assayed as described above. For TI2 and TI3, trypsin inhibitor activity was measured, while chymotrypsin inhibitor activity was measured for TI4, TI5 and TI6. For thermostability assays, KTIs were incubated from 10-100°C at 10°C intervals for 30 min,

then cooled on ice for approx. 30 min before measuring residual PI activity. For TI2 and TI3 thermostability, experiments were also performed with gradual cooling after heating, since this has been shown to improve thermostability (Roychaudhuri et al., 2004), as well as with different incubation times, ranging from 2.5-150 min, but results were the same for both assay adjustments. For stability in the presence of reducing agent, KTIs were incubated with dithiothreitol (DTT) at concentrations increasing exponentially from 1 μM to 100 mM (final concentration) for 30 and 120 min. The reactions were terminated by adding iodoacetamide at twice the concentration of DTT before measuring residual PI activity. For long-term stability, KTIs were stored at 4°C and residual PI activity was measured at 25-day intervals for 100 days. For TI2, residual activity was further measured at 50-day intervals for an additional 200 days.

5.3 Results:

Poplar Kunitz trypsin inhibitor family is large and diverse

As part of our functional characterization of the poplar defense response, we sought to further characterize the wound-inducible KTIs. Consistent with predicted roles in poplar-herbivore defense, wounding induces transcript accumulation of several poplar KTIs, including win3 from hybrid poplar and the *win3* orthologs *TI1* and *TI2* from *P. tremuloides* (Bradshaw et al., 1990; Haruta et al., 2001a). In addition, we have previously shown three distinct KTIs to be among the most abundant and most highly induced transcripts during the wound response (Chapter 2; Christopher et al., 2004). The availability of the *P. trichocarpa* genome sequence facilitated a detailed analysis of the KTI gene family. We found that poplar has at least 22 KTIs (Fig. 5-1), but with sequence ambiguities the number could be as high as 30 KTIs. We considered only gene models that were full length, contained the Kunitz motif ([L,I,V,M]-X-D-X_2-G-X_2-[L,I,V,M]-X_5-Y-X-[L,I,V,M]) and had at least one disulfide bond. KTI gene models with nucleotide sequences ≥ 99% identical were considered to be allelic; such sequences typically had synonomous / non-synonomous substitution ratios of 1. For comparison, sequences ≥ 97% identical that were not considered allelic had ratios of approx. 1.5. Phylogenetic analysis revealed that this large KTI family is highly diverse; at the amino acid level, the

Figure 5-1. Phylogeny of Kunitz TI members from the genus *Populus* constructed by neighbor-joining of protein distance.

Kunitz TI sequences were retrieved from the *P. trichocarpa* genome; only full-length TI sequences with a canonical Kunitz motif sharing less than 96% amino acid identity with other sequences and having EST support were included in this analysis. Numbers at branches represent bootstrap support from 2000 replicates (only values > 80% are shown for clarity). Sequences are annotated with the protein ID from the *P. trichocarpa* genome. Sequences annotated as TI3, TI4, TI5, TI6 and TI7 are the putative *P. trichocarpa* orthologs of Kunitz TIs that were previously identified (Christopher et al., 2004; Talyzina and Ingvarsson, 2006). Accessions of annotated TI sequences (GenBank gene index or JGI protein ID from *P. trichocarpa* genome): GWIN3 (gi|20946), WIN3.12 (gi|169460), TI3 (739064), TI4 (555576), TI5 (574326), TI6 (725622), TI7 (697808).

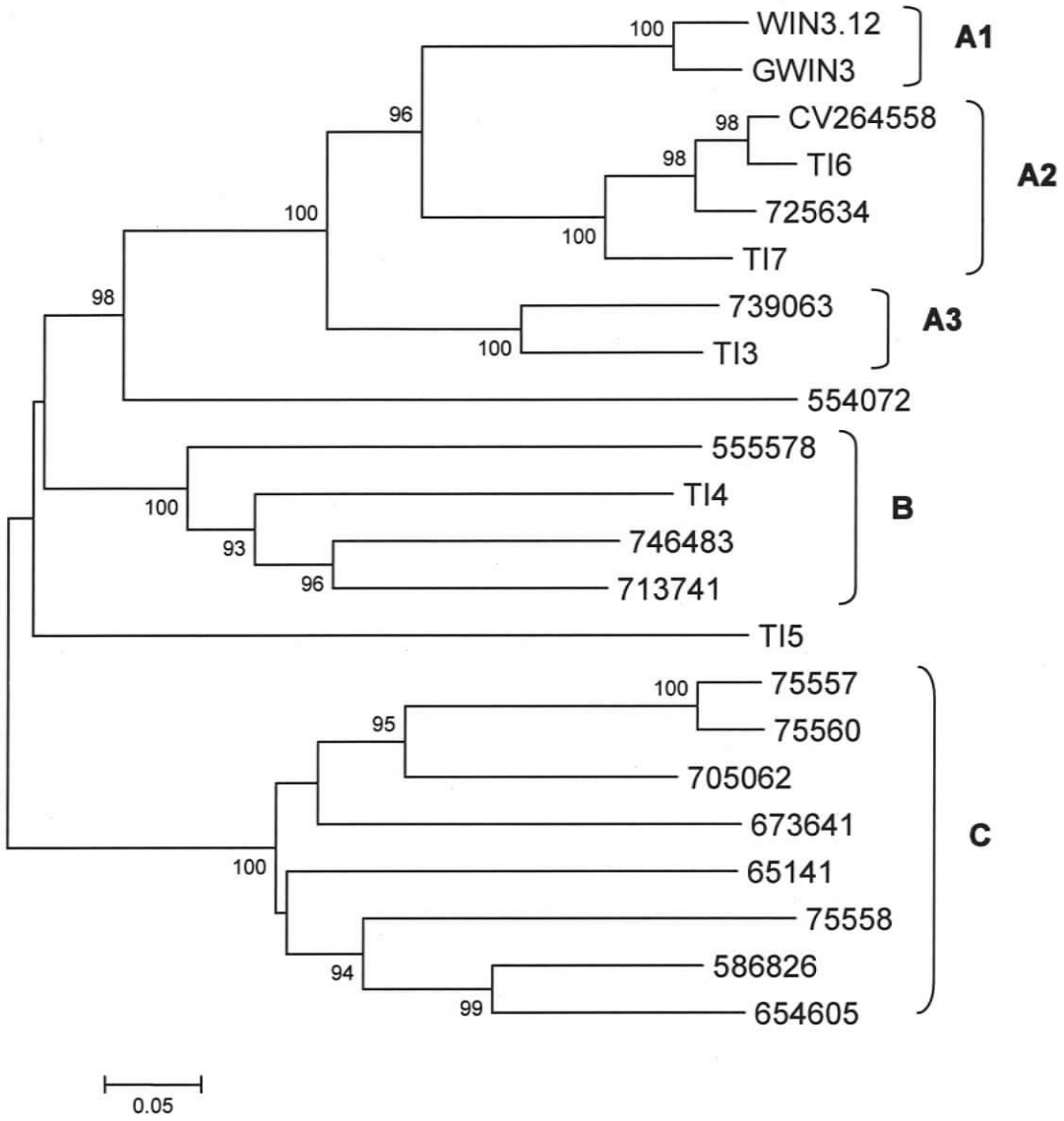

WIN3.12
GWIN3
CV264558
TI6
725634
TI7
739063
TI3
554072
555578
TI4
746483
713741
TI5
75557
75560
705062
673641
65141
75558
586826
654605
A1
A2
A3
B
C
100
96
98
98
100
100
98
100
100
93
96
100
95
100
94
99
0.05

Figure 5-1.

similarity of KTIs members is as low as 25% (Table 5-1). Inspection of this phylogenic tree reveals that the KTI family consists of several clades. KTIs within the same group are approx. 60-70% amino acid similar, while KTIs from different groups are only approx. 30% similar. Moreover, at least one of these homology groups (A) can be subdivided further into subgroups A1, A2 and A3. *GWIN3*, the first poplar TI to be studied (Bradshaw et al., 1990; Hollick and Gordon, 1993, 1995), and *WIN3*-like genes form subgroup A1. This subgroup also includes *PtTI1* and *PtTI2* paralogs from aspen, which we previously suggested to have roles in herbivore defense (Haruta et al., 2001a). We recently identified three new wound-inducible KTIs (*PtdTI3*, *PtdTI4*, *PtdTI5*) from an EST sequencing project and reported their inducible expression profiles (Chapter 2; Christopher et al., 2004). TI3 forms subgroup A3 with 739063, while TI4 is a member of group B with another three genes. TI5 is not part of any homology group and is found on a separate branch. The two recently reported KTIs TI6 and TI7 are part of subgroup A2 with two other genes (Talyzina and Ingvarsson, 2006). Group C includes eight genes, none of which have been previously studied. Together this phylogenetic analysis demonstrates the large size and extensive sequence diversity of the poplar KTI family.

As expected from their sequence identities, a multiple amino acid alignment of representative KTIs from each clade / subgroup illustrates their extensive sequence diversity (Fig. 5-2). This diversity made aligning the sequences somewhat unreliable; to improve the quality of the alignment, secondary and tertiary structure predictions were made using JPred, SWISS-MODEL, CPHmodels and ESyPred3D, and these predictions were used to manually edit and refine the alignment. We also included the extensively studied soybean KTI (STI) and sporamin from *Ipomoea batatas* in the alignment for comparison. While the KTIs have the Kunitz motif, they may otherwise share little overall amino acid similarity, including the position of gaps. However, it is interesting that the most conserved regions correspond to predicted β-sheet formation and for poplar KTIs, the signal sequence. This is consistent with a recent analysis of molecular evolution of the poplar KTI clade A, which showed that the loop regions connecting β-strands are under positive selection (Talyzina and Ingvarsson, 2006). In addition, while some conserved residues are found within the reactive loop of the poplar KTIs, this loop is highly variable, including the P1 residue of the reactive site (boxed area and starred

Table 5-1. Amino acid sequence similarities and identities among members of the poplar Kunitz trypsin inhibitor family[a].

		Amino acid sequence similarity																						
		1	***2***	***3***	***4***	***5***	***6***	***7***	***8***	***9***	***10***	***11***	***12***	***13***	***14***	***15***	***16***	***17***	***18***	***19***	***20***	***21***	***22***	***23***
GmKTI	***1***		22	22	24	24	24	25	23	26	22	27	29	31	30	24	24	24	24	27	26	25	26	27
WIN3.12	***2***	39		93	66	66	69	68	60	56	41	33	34	32	34	30	27	27	30	31	31	29	32	32
GWIN3	***3***	38	96		67	66	70	70	62	57	40	34	35	33	35	31	27	27	30	31	31	29	33	33
CV264558	***4***	40	79	79		97	94	83	62	58	40	35	34	33	35	31	26	26	31	30	31	30	31	31
TI-6	***5***	40	79	79	97		92	81	62	58	40	35	33	32	34	30	26	26	30	29	30	29	30	31
725634	***6***	40	82	82	96	95		88	64	58	42	37	34	33	35	31	26	26	30	31	31	30	32	32
TI-7	***7***	42	83	83	88	88	92		69	62	42	37	33	34	37	33	26	26	31	32	33	30	33	32
739063	***8***	41	74	74	78	77	78	82		77	42	35	36	34	37	31	28	28	30	32	33	29	31	32
TI-3	***9***	41	72	73	76	76	77	79	87		39	34	35	33	35	32	29	28	29	31	32	28	29	31
554072	***10***	39	50	50	51	51	52	53	54	53		31	32	32	33	28	25	26	29	29	32	28	29	29
555578	***11***	43	48	48	49	48	50	52	49	51	47		50	55	55	32	32	31	34	37	36	28	30	30
TI-4	***12***	42	45	46	46	45	46	47	48	47	48	60		60	63	33	30	29	35	31	34	30	31	32
746483	***13***	47	46	46	48	47	48	50	49	48	46	64	69		71	32	35	33	39	37	39	34	35	34
713741	***14***	47	46	46	48	47	49	51	50	49	48	67	71	74		31	32	32	36	35	37	32	33	33
TI-5	***15***	38	43	43	42	42	43	45	43	42	42	46	47	47	46		25	25	31	31	29	26	28	29
75557	***16***	44	40	41	42	42	41	41	42	43	40	43	43	46	45	43		95	67	57	54	59	54	52
75560	***17***	44	40	40	41	41	39	40	41	43	40	43	42	45	44	42	96		68	56	54	59	55	54
705062	***18***	42	38	38	37	37	37	38	39	41	40	42	41	44	45	41	75	75		62	64	56	64	62
673641	***19***	43	40	40	41	40	41	42	42	44	39	44	42	46	46	44	65	66	64		60	54	60	60
65141	***20***	42	40	41	39	39	40	42	40	41	40	42	42	46	45	40	61	61	65	62		56	64	63
75558	***21***	43	41	41	39	39	39	39	39	41	39	40	42	44	43	42	64	65	66	63	59		65	62
586826	***22***	42	38	39	37	37	37	39	38	40	40	41	41	45	44	39	64	64	66	64	63	73		81
654605	***23***	41	40	40	39	38	39	40	40	41	41	39	42	42	42	40	62	63	64	62	63	72	84	
		Nucleotide sequence identity																						

[a]Nucleotide and amino acid sequences were aligned as per the alignment used to generate the poplar KTI phylogeny (Fig. 3), and amino acid similarity or nucleotide sequence identity were calculated by Vector NTI.

Figure 5-2. Protein alignment of representative Kunitz trypsin inhibitor (KTI) sequences from the poplar family.

Soybean KTI (STI) and sporamin of *Ipomoea batatas* (IbSPOA) are shown for comparison. Shading shows conserved (black) and similar (grey) amino acid residues. Sequence gaps are shown by hyphens. Structural features of the proteins are shown. Above the alignment, a solid line marks the putative signal peptides (TI 75560 has no predicted signal peptide), inverted triangles denote the Kunitz motif (L,I,V,M)-X-D-(X_2)-G-(X_2)-(L,I,V,M)-(X_5)-Y-X-(L,I,V,M). Below the alignment, two disulfide bridges formed by four conserved Cys residues are shown. Plus signs denote free Cys residues present in poplar KTIs, except TI5 and 554072. Structural features of STI are also shown, including arrows above the alignment (<—>) delineating b-sheets, a boxed region surrounding the reactive loop and stars which denote the P1 and P1' reactive-site residues. Sequences were retrieved from NCBI with the following accession numbers: STI (1AVU), IbSPOA (Q40084), WIN3 (CAA33539), TI2 (AAK32690), TI3 (AAQ84216), TI4 (AAQ84217), TI5 (AAQ84218) and TI6 (DT502517). Accessions for TIs 554072 and 75560 are JGI protein IDs. TI2 is the predicted *P. tremuloides* ortholog of GWIN3. Poplar TI2, TI3, TI4, TI5 and TI6 were used for heterologous expression of recombinant proteins.

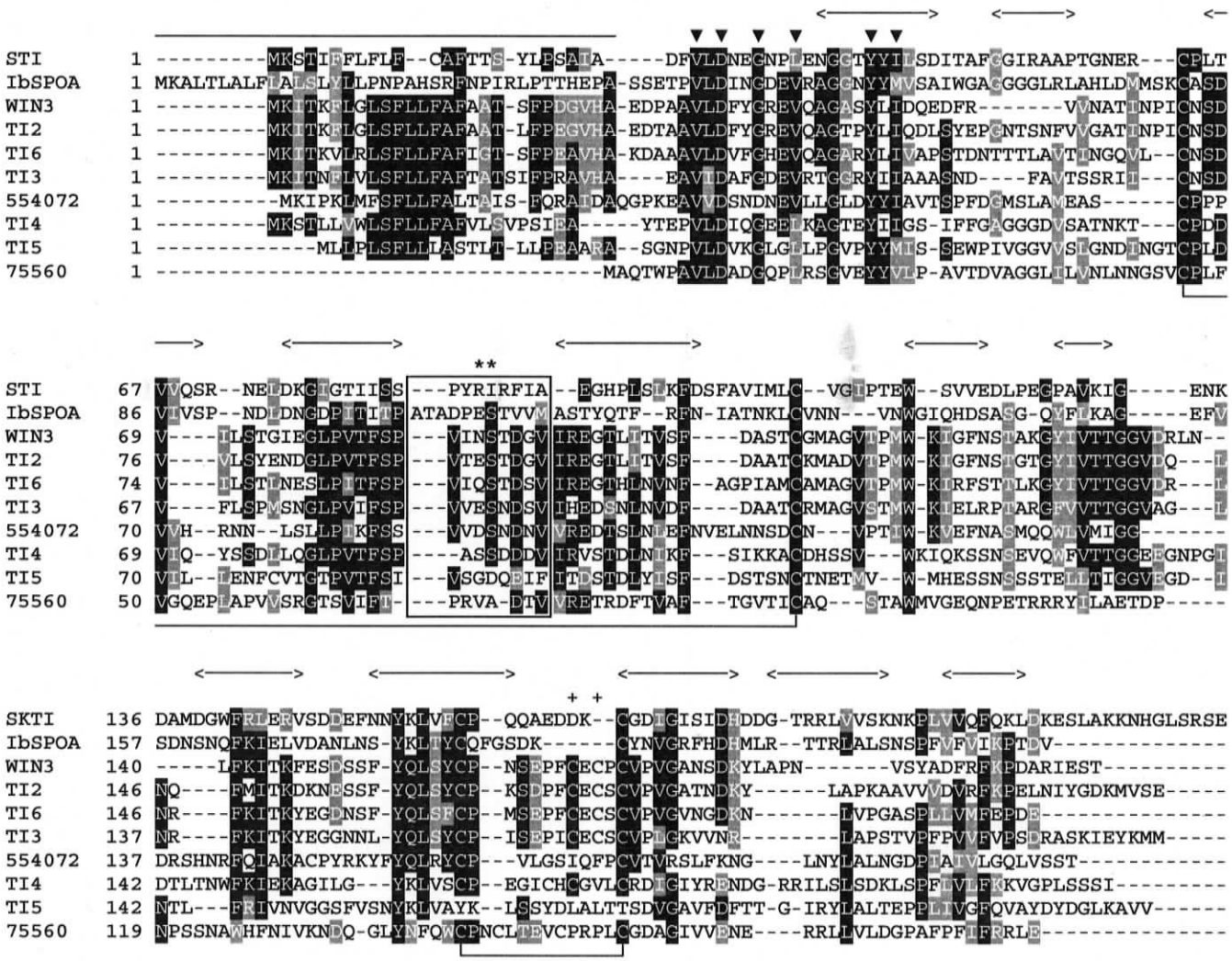


Figure 5-2.

residues, Fig. 5-2). The reactive loop of sporamin has atypical residues compared with STI and other plant KTIs, but is similar to the poplar inhibitors. Among the seven aligned poplar KTIs, six different residues are predicted for the P1, but none are Lys or Arg which are common among plant KTIs and specific for trypsin inhibition (Beynon and Bond, 1989). Interestingly, the entire predicted reactive loops are devoid of Lys or Arg, except KTI 75560. By contrast, most of the poplar KTIs have a Ser as the P1' residue. These conserved and variable regions are presumably indications of the evolutionary pressures exerted on the KTI family (see Discussion).

The poplar KTIs have four cysteine residues that form two conserved intramolecular disulfide bonds, except TI5 which has only two Cys residues that form the first disulfide bond. Interestingly, all the poplar KTIs, except TI5 and 554072, also have two additional free Cys residues located in a loop (Cys165 and Cys167, *win3* numbering). Overall, the poplar KTIs share very little similarity with STI (less than 25% similarity), sporamin and other characterized plant KTIs. In addition, the sequence diversity of the poplar KTIs, especially of the reactive loop, predicts that these proteins likely have distinct inhibitory activities.

Developmental and wound-inducible expression patterns of poplar KTIs

To estimate where the various KTI genes are expressed, we queried the PopulusDB database for digital expression profiles derived from the abundance of ESTs generated from 18 different tissues and developmental stages (Sterky et al., 2004). The expression patterns varied widely across the family (Fig. 5-3), suggesting functional specialization rather than redundant functions for KTIs. We note that digital northerns do not distinguish the expression patterns of highly similar sequences due to ambiguous EST clusters that correspond to several genome models. For example, KTIs TI3 and 739063, which have 87% nucleotide sequence identity (Table 5-1), hit EST sequences from the same cluster in PopulusDB, and so their expression patterns are reported as identical. The highest levels of expression (EST abundance) were found for TI3 / 739063. These were expressed in essentially all tissues, while expression of other KTIs was restricted to only some tissues. KTIs of the A1 and A2 subgroups (e.g. GWIN3, TI6, TI7) were abundant in flowers, young leaves and the apical shoot. None of the remaining KTIs appeared to be

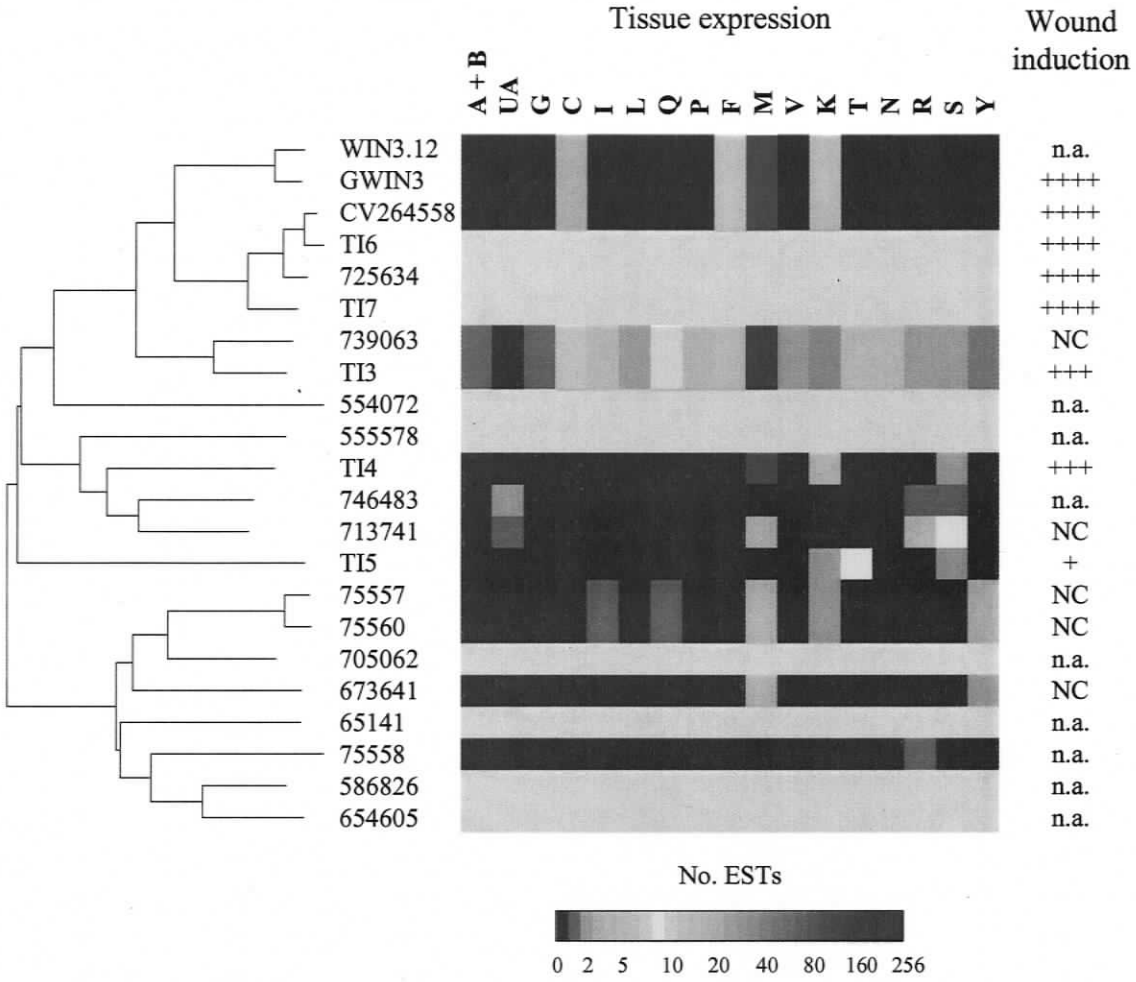


Figure 5-3. Digital northern of expression data for the KTI proteins.

The PopulusDB database (http://poppel.fysbot.umu.se/) was queried for the different KTIs and the number of ESTs from the corresponding cluster are shown (grey = no similar sequence in PopulusDB). For wound induction, data from Christopher et al., 2004 and Ralph et al., 2006 are reported ("+" for degree of induction, "-" symbols for repression, and "NC" for no change of expression). Sequences are annotated as per Fig. 5-1 and the KTI phylogeny at left shows the relationships among the different KTI members. Tissue abbreviations are: A + B, cambial zone; UA, dormant cambium; G, tension wood; C, young leaves; I, senescing leaves; L, cold stressed leaves; Q, dormant buds; P, petioles; F, flower buds; M, female catkins; V, male catkins; K, apical shoot; T, shoot meristem; N, bark; R, roots; S, imbibed seeds; Y, virus/fungus-infected leaves.

expressed at significant levels in healthy leaf tissue, from which most libraries are derived. Interestingly, a large proportion of the KTIs were expressed in floral tissues, most notably female catkins. Since the tissues and EST sets comprising this digital northern did not include herbivore- or stress-induced tissues, we also summarized results from recent transcript profiling studies (Chapter 2; Christopher et al., 2004; Ralph et al., 2006a) (right panel, Fig. 5-3). Inducible expression of leaves by herbivory or simulated herbivory also varies across the family; while most of the KTIs comprising group A are induced by wounding, the remaining KTIs, except TI4 and TI5, do not appear to show this pattern. However, we note that there is no wound-induced expression data for some genes.

To determine whether wound-induced changes in transcript levels translate into increased protein accumulation, we performed western blot analysis with antibodies available for two KTIs. The TI2 antibody is specific for WIN3-like proteins and detected several isomers in leaves (Fig. 5-4). This is consistent with the organization of the *win3* multigene family (Bradshaw et al., 1990; Hollick and Gordon, 1993). We also detected multiple TI2 isoforms in *P. tremuloides* (Haruta et al., 2001a), though fewer than here. A survey of different tissues demonstrated that TI3 was present in leaves, bark, wood and roots, while TI2 was only found in leaves (Fig. 5-4A), which is consistent with the digital northern data (Fig. 5-3). In addition, TI2 protein accumulation was strongly induced by wounding while TI3 protein showed only a modest induction. However, TI2 protein increase was clearly strongest in younger leaves, while accumulation of TI3 protein was relatively consistent regardless of tissue type or age. TI2 and TI3 thus have distinct patterns of protein accumulation, and these patterns are consistent with transcript accumulation. Furthermore, TI2 and TI3 proteins accumulated rapidly after wounding, since no increase in protein was visible within 24 hrs, but both proteins accumulated to maximal levels 2 d after wounding, and levels remained maximal even 7 d after wounding (Fig. 5-4B). We previously showed similar TI2 protein accumulation kinetics in *P. tremuloides* (Haruta et al., 2001a).

Since both transcript and protein levels of poplar KTIs appear to increase in response to wounding, corresponding proteinase inhibitor activity should also increase in leaves. We therefore assayed inhibition of several serine proteinases with leaf extracts

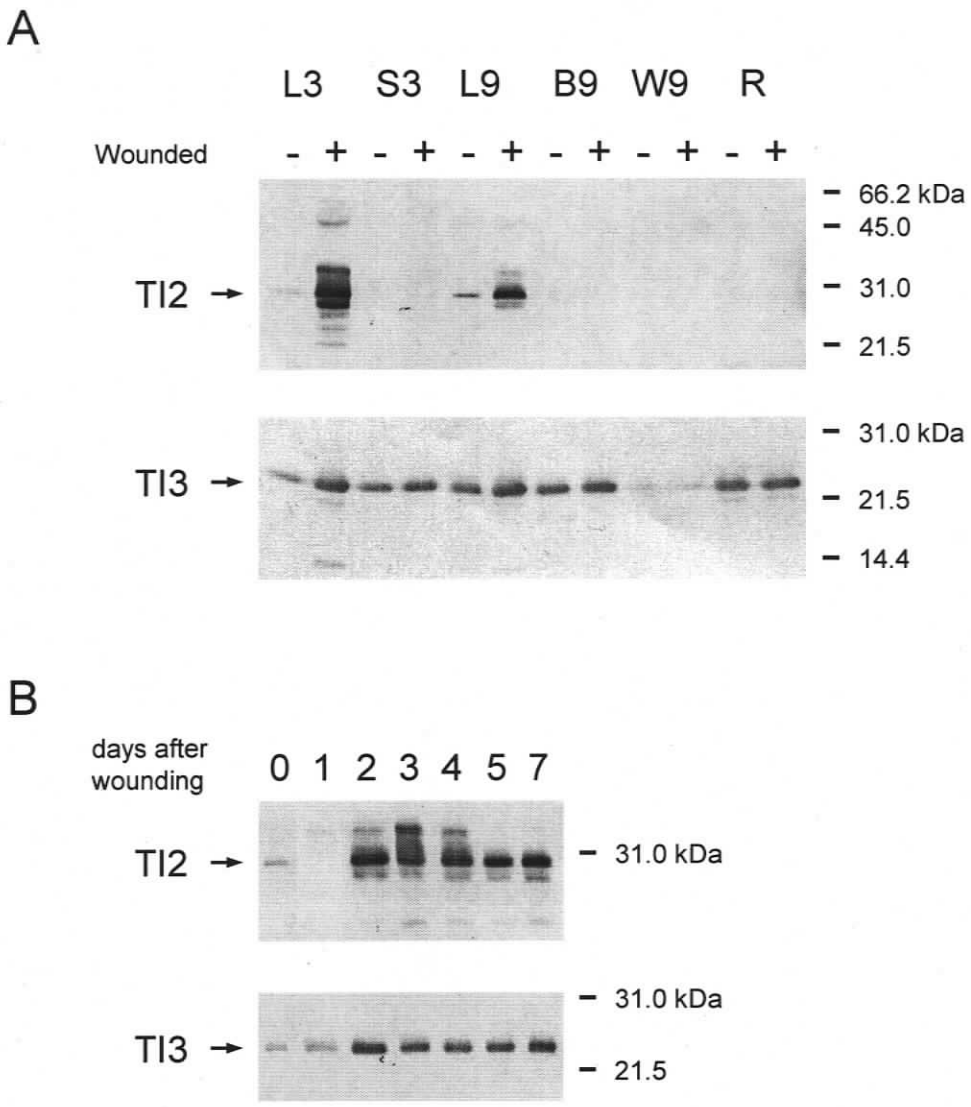


Figure 5-4. Western analysis of TI protein accumulation following wounding of hybrid poplar.

A. Leaves were wounded with pliers and after 4 days tissues were harvested from leaves 3-5 (L3), stem between internodes of leaves 3-5 (S3), leaves 9-11 (L9), bark between internodes of leaves 9-11 (B9), wood between internodes of leaves 9-11 (W9), and mature roots (R).

B. Sapling leaves were wounded with pliers and leaves 9-11 were harvested at the indicated days after wounding.

using kinetic assays; increasing amounts of leaf extract were incubated with trypsin, chymotrypsin or elastase to quantify inhibitor activities. We first compared trypsin inhibitor activity of leaf extracts from young, immature leaves of LPI 3-5 with mature leaves of LPI 9-11 and found that the activity was greatest for younger leaves of both untreated and wounded saplings (Fig. 5-5A and data not shown). We therefore focused on young leaves (LPI 3-5) for tests of chymotrypsin and elastase inhibitor activity. For untreated saplings, leaf chymotrypsin inhibitor activity was highest (IC_{50} = 0.072, Fig. 5-5B), while elastase inhibitor activity was a small fraction of both chymotrypsin and trypsin inhibitor activities. All three proteinase inhibitor activities increased significantly after wounding (2.5 - 5.9X fold change in IC_{50}, $P << 0.001$, Fig. 5-5). The wound-induced increase of trypsin inhibitor activity was the largest, such that for wound-induced saplings, chymotrypsin and trypsin inhibitor activities were comparable. As observed in untreated leaf extracts, elastase inhibitor activity after wound-induction was a fraction of that for trypsin and chymotrypsin. Overall, our finding that leaf inhibitor activity increases after simulated herbivory suggests that induction of TIs is likely a key component of poplar defense induction.

Poplar KTIs exhibit different biochemical properties during protein expression and purification

The substantial (and surprising) sequence diversity of the poplar KTIs is predicted to translate into diverse biochemical and biological properties. To test this hypothesis, we selected TI2, TI3, TI4, TI5 and TI6 as representative of the diversity of the KTI family (Fig. 5-1 and 5-2) for functional characterization by heterologous expression in *E. coli*. We previously reported the expression of recombinant TI2 protein in *E. coli*, and we included this gene for comparison and to expand our previous biochemical analysis. High levels of recombinant protein in *E. coli* were observed for all constructs, with the bulk of the recombinant proteins found in inclusion bodies, as illustrated for TI3 (Fig. 5-6A). The proteins were purified under denaturing conditions and dialyzed in order to re-fold the polypeptides and regenerate active inhibitor proteins. We initially tested the same re-folding conditions used previously for TI2 (Haruta et al., 2001a), but found that most of the other KTIs formed aggregates and precipitated under these conditions. We therefore

Figure 5-5. Inhibitor activity of poplar leaf extracts against various commercial proteases.

Increasing amounts of crude extracts from young leaves (LPI 3-5) of unwounded, control plants (×) and wounded plants (4-days after wounding, ♦) were titrated against (**A**) trypsin, (**B**) chymotrypsin, and (**C**) elastase, and the residual protease activity was measured. Error bars are standard error. Insets show the concentration of leaf extract required to inhibit 50% of the protease activity (IC_{50}).

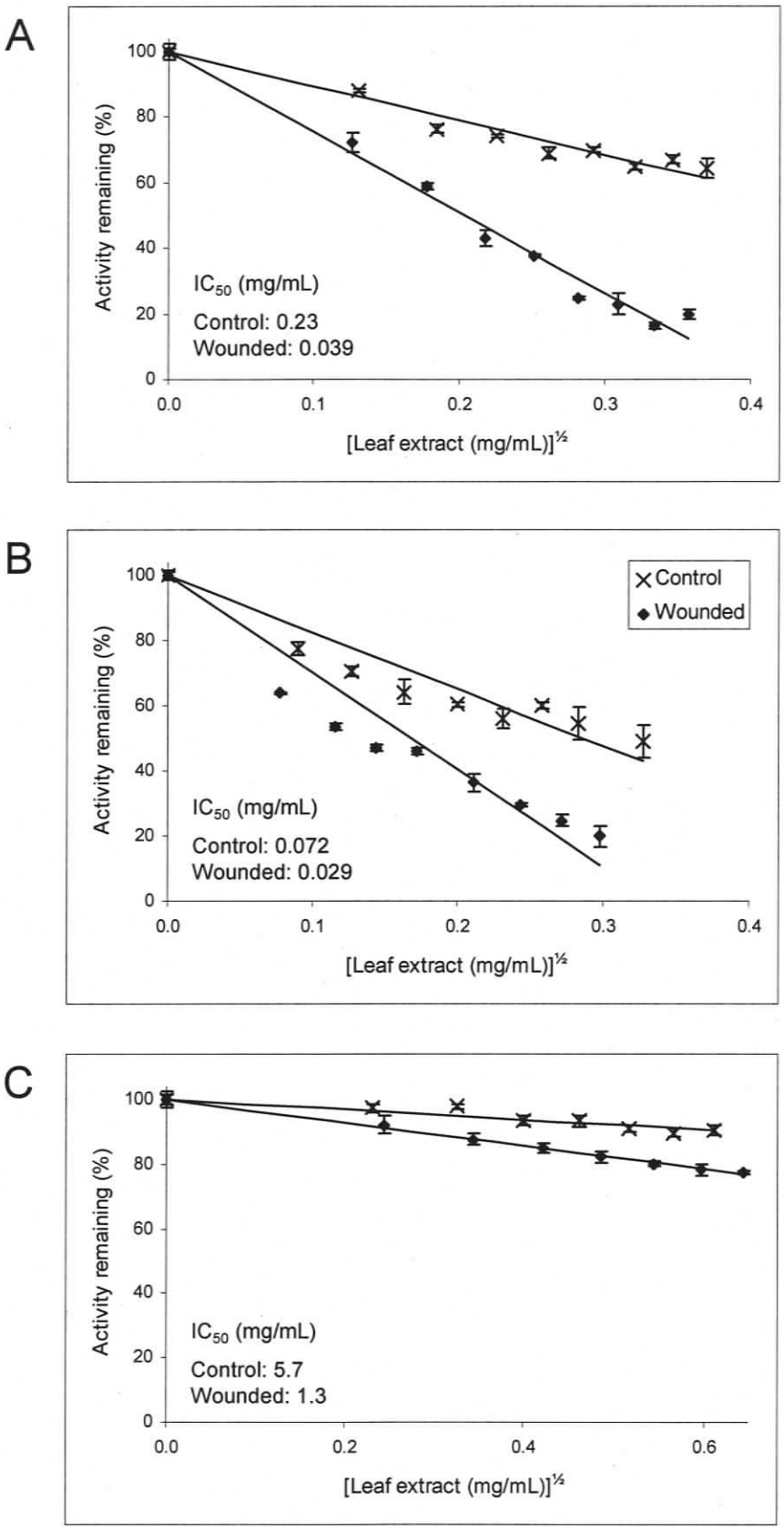

A
Activity remaining (%)
100
80
60
40
20
0
0.0
0.1
0.2
0.3
0.4
IC50 (mg/mL)
Control: 0.23
Wounded: 0.039
[Leaf extract (mg/mL)]½
B
Control
Wounded
Activity remaining (%)
IC50 (mg/mL)
Control: 0.072
Wounded: 0.029
[Leaf extract (mg/mL)]½
C
Activity remaining (%)
0.6
IC50 (mg/mL)
Control: 5.7
Wounded: 1.3
[Leaf extract (mg/mL)]½

Figure 5-5.

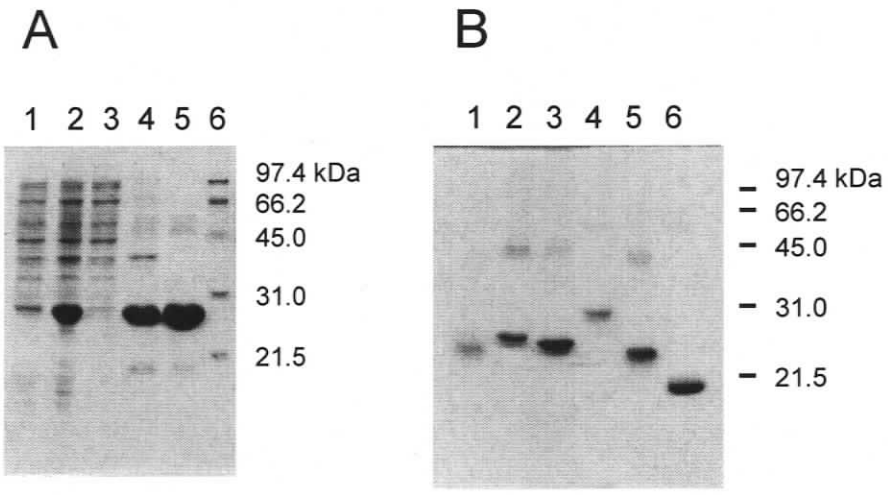


Figure 5-6. Purification of recombinant TIs in *E. coli*.

A. SDS-PAGE analysis of different stages of purification of recombinant TI3 from *E. coli*. Lane 1 - *E. coli* cells before induction with IPTG; lane 2 - *E. coli* cells 24-h after induction with IPTG; lane 3 - *E. coli* cell lysate; lane 4 - lysed inclusion bodies containing TI3; lane 5 - TI3 after purification on Ni-NTA resin column; lane 6 - marker. **B.** SDS-PAGE of recombinant TI2 (lane 1), TI3 (lane 2), TI4 (lane 3), TI5 (lane 4), TI6 (lane 5), and Soybean TI (lane 6).

optimized renaturation conditions by adjusting ionic strength, pH and reducing agent of the buffer; interestingly, these tests showed that optimal conditions varied considerably among the different KTIs (Table 5-2). Thus, four of the KTIs were found to be soluble only in a buffer of high ionic strength, whereas TI3 was soluble in water or a buffered low-strength ionic solution. Moreover, while most KTIs were soluble near pH 8, their solubility differed greatly away from pH 8.

We verified the purity and quantity of the successfully renatured KTIs by SDS-PAGE, which clearly showed a single major band for each KTI at approximate predicted MWs (Fig. 5-6B). In addition, larger bands were apparent for TI3, TI4 and TI6; these may be protein dimers, as the apparent molecular weights of these bands are twice that of the predominant bands. We previously reported putative dimers for TI2 (Haruta et al., 2001a), and other plant KTIs form dimers as well (Terada et al., 1994). The dimers may be linked by an intermolecular disulfide bond formed between the free Cys residues of the KTIs (Fig. 5-2), since the predicted 3D models suggest that the residues are exposed on the surface of the proteins (data not shown). While migration of TI2, TI4 and TI6 in gels corresponded well with their predicted weights (Table 5-2), TI3 and TI5 migrated slower than expected. This may be the result of residual secondary protein structure, since both recombinant proteins migrated precisely according to their predicted molecular weights on denaturing Urea-SDS-PAGE gels, which denature proteins more completely (data not shown).

The predicted poplar KTIs are active proteinase inhibitors and are biochemically distinct

We confirmed that the recombinant and renatured poplar KTI proteins have protease inhibitor activity using *in vitro* assays with commercially available proteases. STI was included for comparison since its activity has been extensively studied. Because previous studies of plant KTIs have found a wide range of possible target proteases (see section 5.1), we assayed inhibitor activity against a variety of proteases: the serine proteinases trypsin, chymotrypsin, elastase and subtilisin, and the cysteine proteinase papain. Considering our previous findings that TI2 was a strong inhibitor of trypsin, we used kinetic assays at multiple TI:protease ratios to accurately test inhibition of trypsin,

Table 5-2. Properties of recombinant poplar trypsin inhibitors.

TI	Size (aa)	Molecular weight (kDa)		Predicted pI	Solubility	
		Predicted	Actual [SDS-PAGE]		water	50 mM Tris (pH range)
TI2	213	23.1	24	4.42	insoluble	2; 5 – 12
TI3	202	22.0	26	6.15	soluble	2 – 4; 9 – 12
TI4	212	23.1	24	4.96	insoluble	2 – 3; 8 – 12
TI5	214	22.9	30	3.98	insoluble	7 – 12
TI6	202	21.8	23	5.32	partially soluble	2 – 3; 7 – 12

chymotrypsin and elastase. This assay design is more accurate than end-point assays, since activity measurements are effectively the mean of continuous time points. For proteinases outside the chymotrypsin subfamily of serine proteinases, we used end-point assays for preliminary tests of subtilisin and papain inhibition. The experiments confirmed that all KTIs tested are active inhibitors of at least one of the proteinases tested. However, the individual inhibition profiles were remarkably different (Fig. 5-7). For example, TI2 was the strongest inhibitor of trypsin (Fig. 5-7A), with activity levels similar to that of STI, while TI3, TI5 and TI6 had intermediate levels of activity. In contrast, TI4 had no trypsin inhibitory activity. In chymotrypsin inhibition assays (Fig. 5-7B), TI6 was the strongest inhibitor among the poplar TIs, though it was less potent than STI, followed by TI3, TI5, and TI4 in decreasing order. While TI2 had the most trypsin inhibitor activity, it showed almost no inhibition against chymotrypsin. By contrast, the only poplar KTI with inhibitory activity against elastase was TI6 (Fig. 5-7C), with stronger activity than STI. Therefore at least one poplar KTI was a potent inhibitor of trypsin, chymotrypsin, and elastase, and often several were very effective. However, none of the poplar KTIs tested were active against subtilisin or papain (Table 5-3).

To directly compare the inhibitory activities of the KTIs, we calculated the concentration at which each KTI inhibits 50% of proteinase activity (IC_{50}) (Table 5-3). This comparison clearly shows that the five poplar KTIs have very different inhibitory activity profiles. Moreover, a comparison of IC_{50} values indicates that many KTIs have strong preferences for specific proteinase targets (Table 5-3). For example, TI2 appears to be a specific inhibitor of trypsin, while TI4 was specific to chymotrypsin. TI3 and TI5 both inhibited trypsin and chymotrypsin, and TI6 was the strongest inhibitor of chymotrypsin and elastase. Together, the results from these assays illustrate that the poplar KTI genes all encode functional proteinase inhibitor proteins, but that they are functionally specialized.

Poplar KTIs are stable proteins but have different properties

KTIs from other plants have often been reported to be exceptionally stable proteins. We thus investigated whether the poplar KTIs are stable in the presence of a reducing agent or increasing temperature. For assays of stability in the presence of a

Figure 5-7. Inhibitory activites of recombinant poplar TIs against various commercial proteases.

Titration of increasing amounts of TI-2 (blue diamond), TI-3 (red square), TI-4 (purple triangle), TI-5 (orange closed circle) and TI-6 (green 'x') against (**A**) trypsin, (**B**) chymotrypsin, and (**C**) elastase. Soybean TI (yellow dash) is shown for comparison as a positive control and bovine serum albumin (BSA; indigo open circle) is shown as a negative protein control. Error bars are standard error.

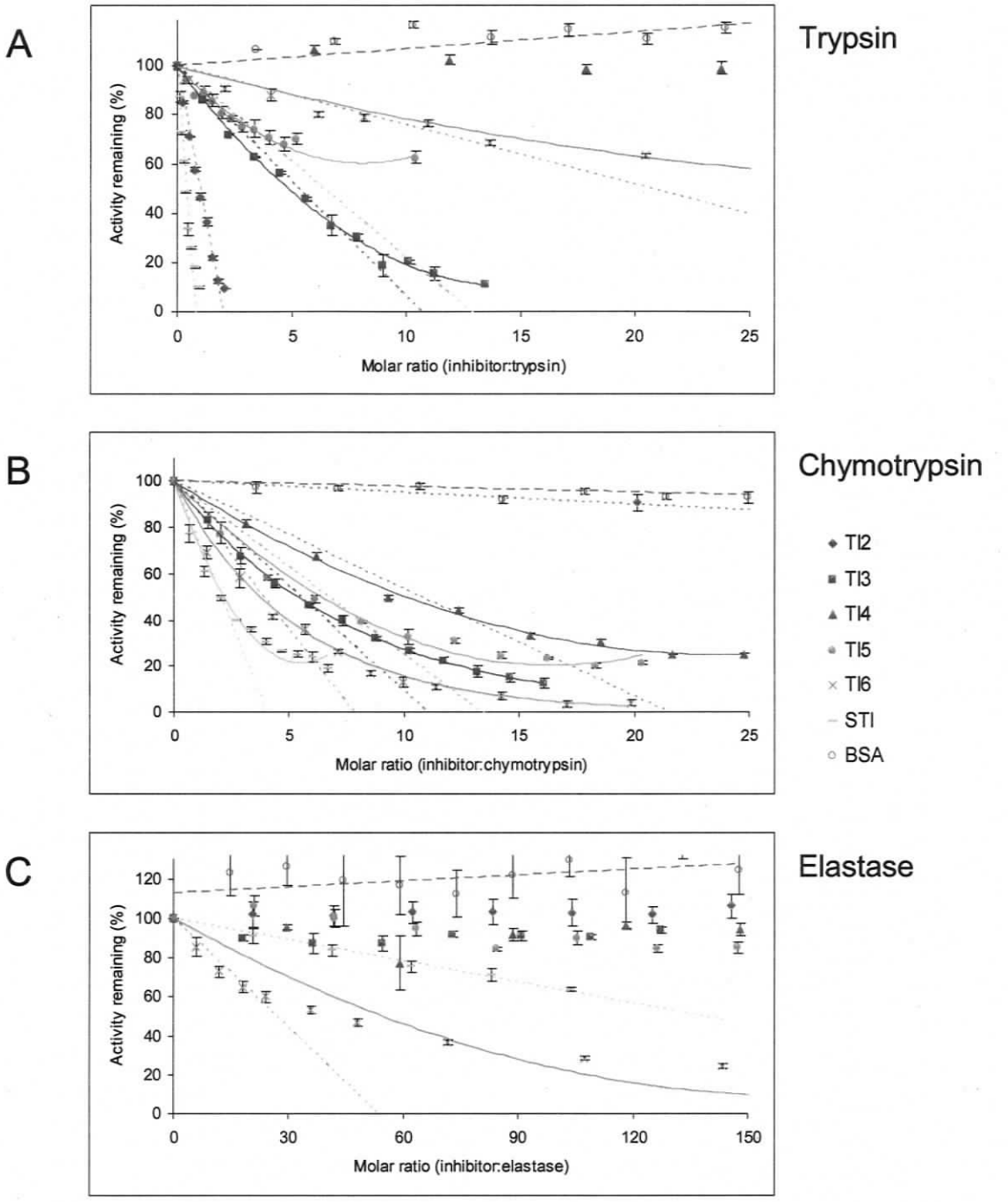

A
Trypsin
Activity remaining (%)
Molar ratio (inhibitor:trypsin)
B
Chymotrypsin
Activity remaining (%)
Molar ratio (inhibitor:chymotrypsin)
TI2
TI3
TI4
TI5
TI6
STI
BSA
C
Elastase
Activity remaining (%)
Molar ratio (inhibitor:elastase)

Figure 5-7.

Table 5-3. Summary of inhibitory activities of KTIs against different proteases.

	50% inhibitory concentrations (μM)				
	Trypsin	Chymotrypsin	Elastase	Subtilisin	Papain
TI2	0.0280	2.97	NA	NA	NA
TI3	0.152	0.153	14.9	NA	NA
TI4	128	0.294	NA	NA	NA
TI5	0.205	0.190	1.84	NA	NA
TI6	0.643	0.122	0.147	NA	NA
STI	0.0116	0.0571	0.735	NA	NA

NA: no activity; n.d.: not determined

reducing agent, purified recombinant KTIs were incubated in the presence increasing concentrations of DTT, after which inhibitor activity was measured and compared to activity in the absence of DTT. We found that the KTIs were stable in the presence of increasing DTT concentration for 30 mins, with little change up to 2 h (Fig. 5-8A and data not shown); however, each KTI had a distinct stability profile. In general, the KTI proteins were stable to a critical DTT concentration, beyond which they quickly lost inhibitor activity. This critical concentration and the rate of activity loss differed for each KTI (Fig. 5-8A). TI4 and TI6 were sensitive to 0.1 mM DTT but were differentially sensitive beyond this concentration. TI3 retained activity up to 1 mM DTT but quickly lost activity above this level. TI5 was most resistant to reducing conditions, since this KTI retained 65% activity at 100 mM DTT, even after 2-h incubation. Interestingly, TI2 activity increased to an optimal 31.6 μM DTT, but quickly lost activity at higher concentrations and lost all activity beyond 3 mM DTT.

Similarly, we found that the poplar KTIs had different stabilities to elevated temperatures (Fig. 5-8B). KTI proteins were incubated for 30 mins at different temperatures, and then inhibitory activity was measured and compared with activity of KTIs kept on ice. TI2 was most thermolabile, already losing activity at 30°C. Similarly, TI3 was susceptible to temperatures above 40°C. To determine if rapid cooling on ice after heating caused the apparent thermolability of TI2 and TI3, we repeated the experiments with gradual cooling after heating but found similar losses of activity (data not shown). TI6 was more thermostable than both TI2 and TI3, remaining active after incubation at 60°C. None of TI2, TI3 or TI6 were boiling stable, since they retained less than 20% activity after boiling. By contrast, TI5 was stable at all temperatures, retaining 80-100% activity at all temperatures assayed. TI4 was also stable at high temperatures, but interestingly exhibited higher activities of 125-130% when incubated at 60°C and above. In view of the stability of all the KTIs at lower temperatures, we tested long-term stability of poplar KTIs at 4°C by assaying the recombinant KTI preparations at 25-day intervals for 150 days (Figure 5-9A). We found no loss in activity, as the activities of the KTIs fluctuated between 80% and 130%. We extended this analysis to more than one year for TI2, again measuring activity periodically, and found no loss of activity. Overall, our analyses demonstrate the remarkable stability of the poplar KTI proteins. They also

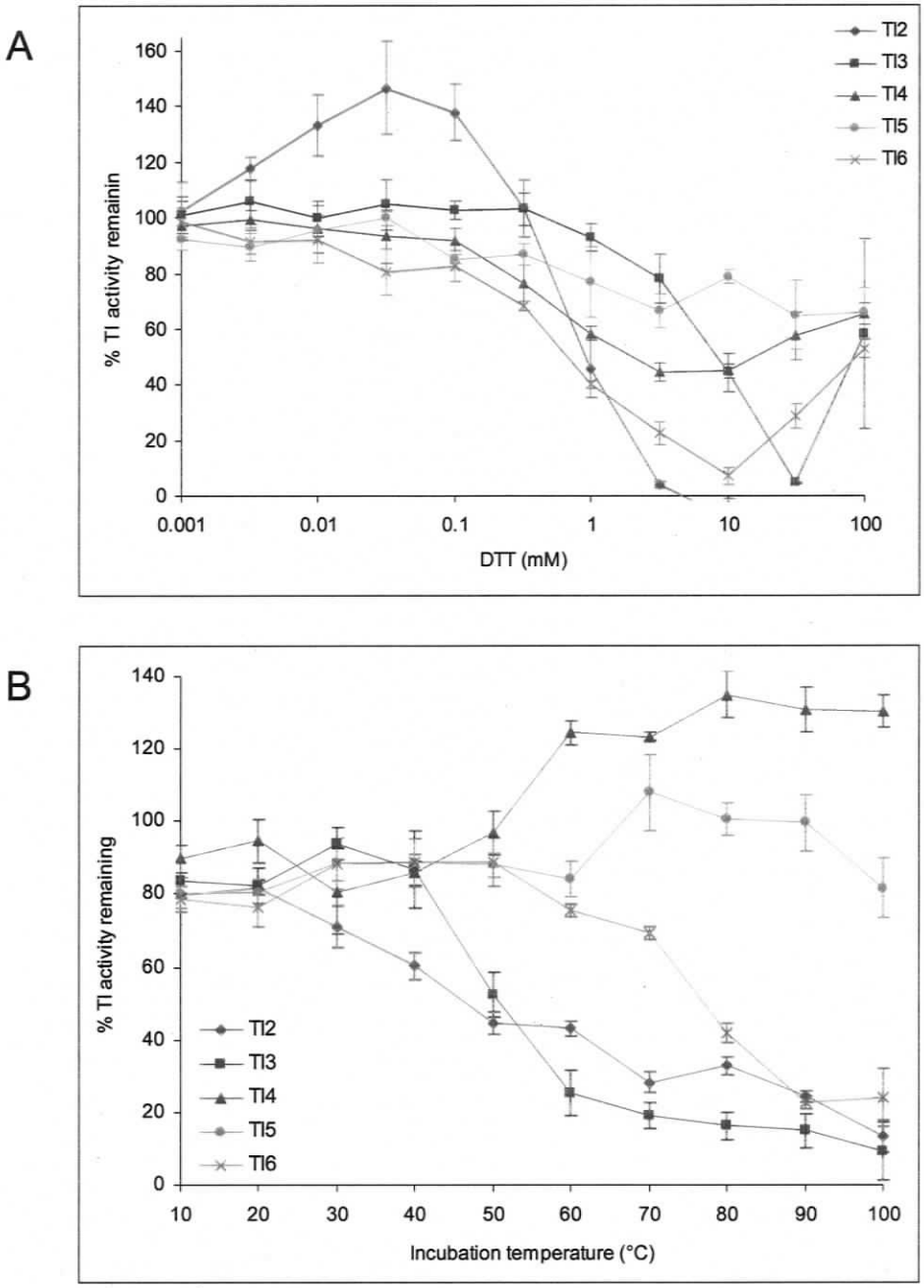


Figure 5-8. Effect of denaturing conditions on activity of poplar TIs.

A. KTIs were incubated in the presence of increasing concentrations of DTT for 30 min followed by incubation in iodoacetamide.

B. KTIs were incubated at increasing temperatures for 30 min followed by incubation on ice. TI2, blue diamond; TI3, red square; TI4, purple triangle; TI5, orange circle; TI6, green 'x'. Error bars are standard error.

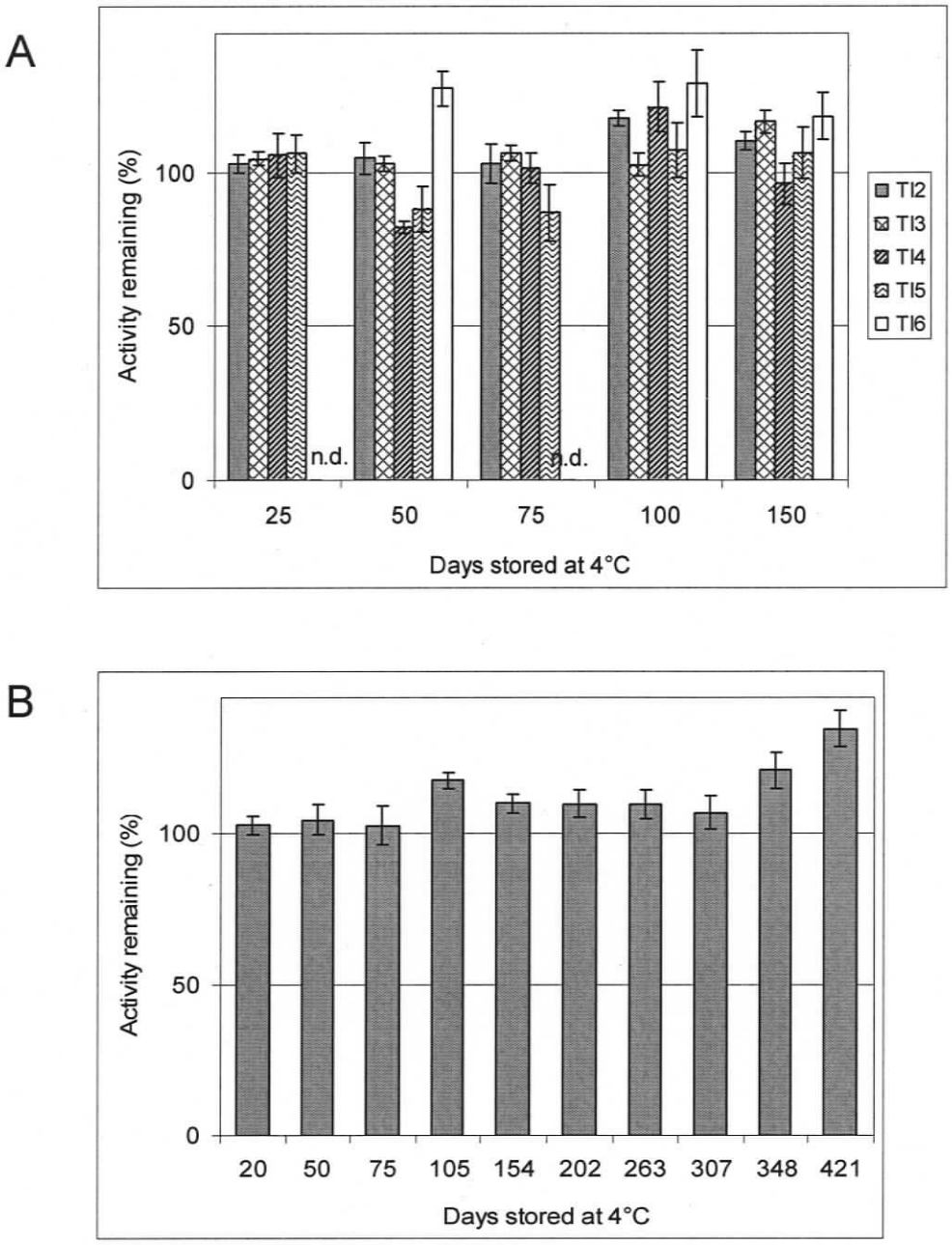


Figure 5-9. Long-term stability of poplar TIs stored at 4°C.

A. Residual activities of poplar TI2 (grey), TI3 (cross-hatch), TI4 (upward diagonal), TI5 (zigzag) and TI6 (white) were measured at 25-day intervals during storage at 4°C for 100 days.

B. Residual activity of TI2 was measured at approx. 50-day intervals during storage at 4°C for an additional 200 days. Error bars are standard error.

emphasize the observation that these are biochemically diverse proteins.

TI3 inhibits larval midgut proteolytic activity of a lepidopteran insect

Although we showed that each poplar KTI was an active inhibitor of at least one type of proteinase tested, none were of insect origin. To determine if the poplar KTIs can inhibit insect pest digestive proteinases, we tested them against midgut extracts from bertha armyworm (BAW, *Mamestra configurata*), a lepidopteran pest of crucifers. The midgut proteases of BAW have been characterized, and like other lepidopteran larvae consist primarily of serine proteinases, particularly trypsin-, chymotrypsin- and elastase-like proteases (Hegedus et al., 2003). For these assays, different amounts of poplar KTIs were incubated with a standard quantity of BAW midgut extract, and the remaining protease activity was measured. Assays were performed at two different pH using an end-point assay with azocasein as a general protease substrate (Hegedus et al., 2003). These two pH assays correspond to two pH optima in protease activity of BAW midguts. In contrast to our results with commercial proteinases, TI3 was the only member of the poplar KTIs tested with activity against BAW midgut proteases (Fig. 5-10A and data not shown). STI was also an effective inhibitor, consistent with the results of Hegedus et al. (2003) (Fig. 5-10B). Both TI3 and STI were more potent inhibitors of BAW gut protease activity at pH 11 than 8. At pH 11, TI3 inhibited approx. 40% of BAW midgut proteolytic activity, while STI inhibited at least 50% of the activity (no maximum inhibition was reached for STI). However, only 0.5 μg of TI3 was needed for 40% inhibition, whereas approx. 6.7 μg of STI was required to attain the same inhibition, indicating TI3 has a much greater affinity for BAW midgut proteases. A similar trend was observed for pH 8 assays, where TI3 was more potent but had a lower maximum inhibition of gut protease activity compared to STI.

5.4 Discussion:

In order to functionally analyze the diverse poplar KTI gene family, we selected herbivory-inducible genes that represent the diversity of the entire family. Our results demonstrate that the five representative poplar KTI genes analyzed encode functional proteinase inhibitors and that each protein analyzed is functionally distinct. However,

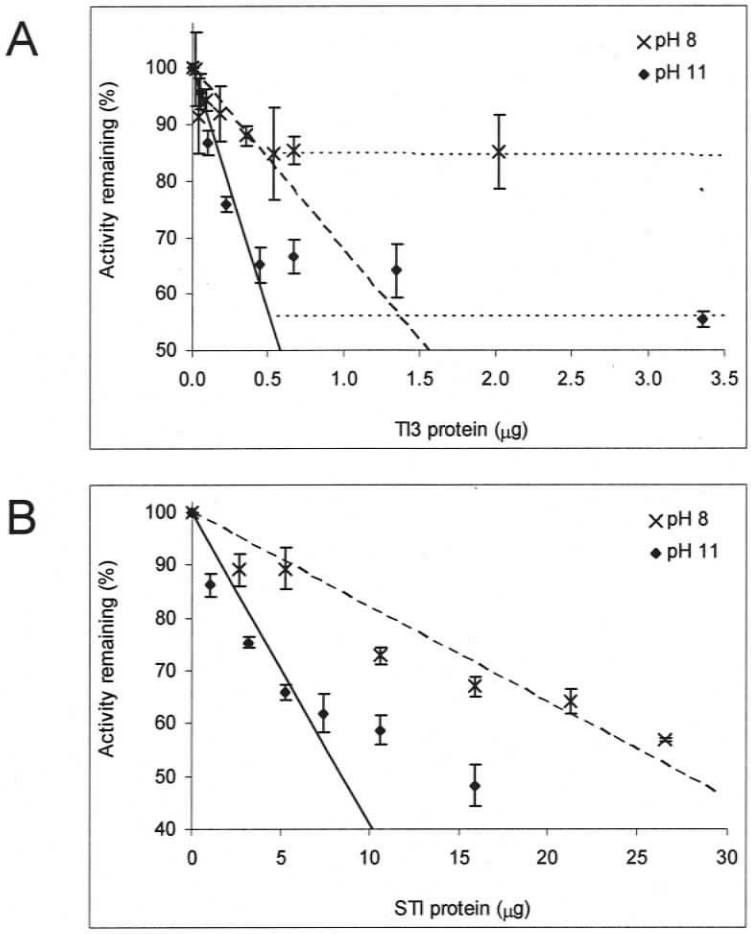


Figure 5-10. Inhibitory activities of poplar TI3 and soybean KTI (STI) against proteases from bertha armyworm (BAW, *Mamestra configurata*).

Increasing amounts of TI3 (**A**) and STI (**B**) were preincubated with a constant amount of BAW midgut extract and the residual total proteolytic activity was assayed at pH 8 and 11 with azocasein as a substrate. The two pH conditions correspond to two major peaks of protease activity in BAW midguts (Hegedus et al., 2003). Error bars are standard error.

only one of the KTIs, TI3, is effective against midgut proteolytic activity of bertha armyworm. In addition, our analyses illustrate the high, yet differential, stability of poplar KTIs, which emphasizes their biochemical diversity.

Poplar Kunitz TIs are functional inhibitors of proteinases

Our results demonstrate that the five poplar KTIs tested here are all active, inhibiting at least one proteinase. This is an important finding since some Kunitz-like proteins have no proteinase inhibitory activity (McCoy and Kortt, 1997) or have other activities such as invertase inhibition or lectin-like activity (Glaczinski et al., 2002; Macedo et al., 2004). Moreover, a potato Kunitz-type PI was recently shown to be a strong chymotrypsin inhibitor, whereas sequence similarity predicted cathepsin D inhibition (Lison et al., 2006). While the poplar KTIs appear to be less effective than STI at inhibiting trypsin and chymotrypsin, we may be underestimating the poplar KTI activity since we studied recombinant proteins renatured from insoluble inclusion bodies of *E. coli*, which may not be completely renatured. Some plant KTIs are post-translationally modified, but previous work strongly suggests that inhibitors expressed in *E. coli* have comparable activity to the native inhibitor (Kouzuma et al., 1997; Iwanaga et al., 1998; de Souza et al., 2005). The recombinant poplar KTIs included terminal peptide tags which were used for purification but could potentially interfere with protein function. However, other studies have measured activity from recombinant plant KTIs with GST-tags, which are much larger (27 kD) than the His or T7 tags (1.5 kD) used here (Saarikoski et al., 1996; Yao et al., 2001; Heibges et al., 2003b). Furthermore, comparative inhibition assays showed that the range of proteases inhibited was not significantly affected by the GST-tag (Heibges et al., 2003b). Therefore, we are confident that our measures of total inhibitory activity, as well as comparative activity profiles against different proteinases, are robust.

Wound-inducibility of KTIs is correlated with inhibitor activity in poplar leaves

Based on our interest in herbivory, we chose to characterize representative poplar KTIs of clades that were wound-inducible. Thus, we did not study group C genes of the poplar family. We used results from published array and northern analyses to deduce that

group C KTIs are apparently not wound-inducible (Fig. 5-3). Furthermore, the inducible nature of TI2 and TI3 proteins was confirmed by western blot analysis, which showed rapid accumulation of both TI2 and TI3 after wounding (Fig. 5-4). The wound-induced accumulation of KTI transcripts and proteins is consistent with our assays of leaf total proteinase inhibitor activity. Leaf extracts have high levels of trypsin and chymotrypsin inhibitor activity but only low levels of elastase inhibitor activity (Fig. 5-5). This is consistent with our functional data of KTIs, since only TI6 inhibited elastase (Fig. 5-7). In general, inhibitor activities of leaf extracts can be correlated with the activities of the KTI proteins studied individually. Thus, poplar leaf extracts inhibited more chymotrypsin than trypsin, consistent with the general preference of chymotrypsin inhibitory activity of poplar KTIs.

Analysis of transcript and protein accumulation patterns with digital northerns and westerns shows diverse expression patterns across the KTI family (Fig. 5-3 and 5-4). For the western analysis, TI2 and TI3 antibodies detect proteins encoded by *win3* and *TI3*, respectively. Comparison of digital northern and western analyses shows corresponding expression patterns in terms of transcript and protein accumulation. TI2 protein accumulated to highest levels in young leaves, consistent with *win3*-like transcripts detected mostly in young leaves and the apical shoot. By contrast, TI3 protein and transcripts accumulated in almost all tissues (Fig. 5-3 and 5-4). In previous work with *P. tremuloides*, we detected TI3 transcripts in leaves, petioles, midveins, bark, wood and roots (Chapter 4 and data not shown). This apparent ubiquitous expression of TI3 is curious, since proteinase inhibitors can incur large fitness costs (Zavala and Baldwin, 2004). By contrast, the remaining poplar KTIs exhibit relatively tissue-specific patterns of expression (Fig. 5-3). This suggests that TI3 has acquired new regulatory sequences during evolution leading to its broad expression.

Poplar KTIs are biochemically and functionally diverse

The poplar KTIs tested inhibit trypsin, chymotrypsin and elastase with different strengths, illustrating their different proteinase substrate preferences and functional diversity. TI2 is the most potent trypsin inhibitor, while TI6 is the most potent inhibitor of chymotrypsin and elastase. Furthermore, TI2 exhibits a strong preference for inhibition

of trypsin and TI4 a strong preference for chymotrypsin, while TI3 and TI5 inhibit trypsin and chymotrypsin with similar efficiency. Such functional diversity within the poplar KTI family is consistent based on their sequence diversity. Studies with other species have also shown that plant KTIs from gene families can have different inhibition profiles and inhibit diverse substrates. Extensive biochemical analysis of the 21-member KTI potato family revealed functional diversity both between and within the three homology groups (Heibges et al., 2003a; Heibges et al., 2003b). Group A and B potato KTIs inhibit trypsin strongly, while group C KTIs do so only weakly. KTIs classified in these homology groups also inhibit other target enzymes, including elastase, subtilisin, the aspartic proteinases cathepsin D and aspergillopepsin, and cysteine proteinases (Ritonja et al., 1990; Rowan et al., 1990; Valueva et al., 2000; Heibges et al., 2003b; Revina et al., 2004).

Interestingly, the functional diversity of the poplar KTIs did not correspond to their sequence diversity. Although TI2 and TI6 were most similar among the KTIs tested (Table 5-1), their inhibitory activities were the most divergent. TI2 strongly inhibited trypsin but not chymotrypsin or elastase, while TI6 was the strongest poplar inhibitor of chymotrypsin and elastase but only marginally inhibited trypsin (Fig. 5-7). TI6 was the only elastase inhibitor, which is a rare activity among plant KTIs (Valueva et al., 2000; Araujo et al., 2005; Sumikawa et al., 2006). In contrast, TI3 and TI5 inhibited proteinases with similar potencies, but have little sequence similarity. Comparison of reactive site and reactive loop residues among the KTIs also reveals that these amino acid sequences do not group according to proteinase substrate preferences. This is consistent with the atypical residues of the poplar KTI reactive loops (Fig. 5-2). The putative reactive site and reactive loop of the KTIs predicted by our work are based on overlayed secondary and tertiary structure predictions as well as sequence alignments with other plant KTIs. This is likely why our predictions of the KTI reactive loops differ from previous studies of *Populus* and *Salix* KTIs (Saarikoski et al., 1996; Talyzina and Ingvarsson, 2006). A similar approach has been used to predict the second reactive site of a winged bean chymotrypsin inhibitor (Dattagupta et al., 1999).

Unlike poplar KTIs, reactive site residues of other plant KTIs may be good indicators of inhibitor specificity. For example, Arg / Lys and Ile / Ser are often the

reactive site residues for potent inhibitors of trypsin, while Leu-Ser is often the reactive site bond for KTIs effective against chymotrypsin. However, some plant KTIs resemble the poplar KTIs in that they also have atypical reactive site residues. Glu-Ser are the reactive site residues of DrTI from *Delonix regia* seeds and sporamin from *Ipomoea batatas* tubers (Pando et al., 2001; Yao et al., 2001), and are also the predicted residues for TI2 and TI3 reactive sites. Furthermore, site-directed mutagenesis of the sporamin reactive site from Glu to Arg or Ser to Ile completely abolishes trypsin inhibitor activity (Yao et al., 2001), despite the presence of these residues in many trypsin inhibiting KTIs. Mutation of another negatively charged residue in the sporamin reactive loop also abolishes inhibitory activity (Yao et al., 2001), indicating that negatively charged residues in the sporamin reactive loop may be responsible for protease inhibitory activity. Interestingly, 19 of 22 poplar KTIs have a conserved negatively charged residue in their reactive loops. Thus, the interactions between poplar KTIs and substrate proteinases may be similar to the situation in DrTI and sporamin.

We also found that the biochemical properties of the poplar KTIs differed considerably. Consistent with other studies of plant KTIs, several poplar KTIs are stable in the presence of the reducing agent DTT as well as at high temperature (Garcia et al., 2004). However, these stability profiles varied greatly among the five poplar KTI proteins. In some cases, high temperatures and DTT enhanced inhibitor activity; TI4 activity increased after heating and TI2 activity increase after DTT incubation, possibly the result of incomplete renaturation. However, previous plant KTI studies based on re-folded proteins from inclusion bodies found that the activity of renatured proteins is comparable to activity of native inhibitors (Kouzuma et al., 1997; Iwanaga et al., 1998). In our assays, TI5 was the most stable KTI, retaining activity at high levels of DTT and high temperature, while TI2 was most sensitive, losing one-half of its activity at 1 mM DTT and 50°C (Fig. 5-8). The presence of only a single disulfide bond may contribute to the high stability of TI5, at least in the presence of DTT. Although several KTIs have been identified that have one or no disulfide bonds, their stability is not reported. Studies of other plant KTIs also reveal diverse stability profiles. Some KTIs are exceptionally resistant to reducing agents, boiling and pH extremes (Garcia et al., 2004), while others are sensitive (Macedo et al., 2004).

Poplar KTI properties are consistent with a role in herbivore defense

High protein stability may be related to the herbivore defense roles of plant KTIs, since the harsh environment within herbivore digestive systems necessitates intrinsic protein stability. Recently, the tomato anti-herbivore enzymes arginase and threonine deaminase, were shown to be stable in midguts of *Manduca sexta* and to catabolize essential amino acids resulting in decreased insect growth (Chen et al., 2005; Chen et al., 2007). This supports the idea that anti-herbivore proteins may be inherently more stable and resistant to proteolysis. We therefore tested the stability of poplar KTIs in the guts of forest tent caterpillar (FTC; *Malacosoma disstria*), a natural herbivore of the *Populus* genus. Both TI2 and TI3 are intact in FTC frass. Western analysis showed that frass from FTC larvae fed control and induced poplar foliage contained intact fragments of both TI2 and TI3 (Supplemental Fig. 5-1). This suggests that poplar KTI stability may confer resistance to conditions within the guts of insect herbivores. Consistent with poplar KTIs having anti-herbivore properties, we show that TI3 is an active inhibitor of BAW midgut proteases, and for those proteases sensitive to TI3-inhibition, TI3 is a more potent inhibitor than STI (Fig. 5-10). This confirms that at least one poplar KTI has anti-insect properties and supports a role for the KTIs in poplar defense.

Overall, our results provide insights about the tremendous diversity of poplar KTIs. Clearly, the sequence diversity of the KTI family translates to biochemical and functional diversity. Molecular evolution analyses have shown that poplar KTIs comprise a rapidly evolving gene family (Ingvarsson, 2005; Talyzina and Ingvarsson, 2006), with some KTIs under strong selection pressures. These results should aid future analysis of the evolution poplar KTIs by clarifying how evolved sequence changes affect inhibitor function. Moreover, our results support the idea that gene duplications followed by strong positive selection has led to functional specialization of KTIs. The purpose for such strong selection pressures and functional diversity of the KTI family is not yet proven, but is predicted to be due to insect herbivore pressures from a diversity of herbivore pests and multiple digestive proteinases. The KTI family inhibits different proteinase substrates and may provide defense against a wide range of pests. Since poplars are long-lived trees, this large and diverse family may be crucial for survival in time and space against multiple pest species with short generation times capable of quick evolutionary changes.

6 A poplar Kunitz-type trypsin inhibitor inhibits digestive proteases, reduces growth, and elicits hyperproduction of digestive enzymes in forest tent caterpillar (*Malacosoma disstria*)

[The following chapter is in preparation for submission to Insect Biochemistry and Molecular Biology]

6.1 Introduction

Insect herbivores encounter numerous defenses when feeding on plant tissues, which may be constitutive or induced in response to feeding. Inducible defenses include both proteins and secondary metabolites that act as toxins, antifeedants, or antinutrients (Walling, 2000; Gatehouse, 2002). However, insects have evolved mechanisms to avoid, tolerate or detoxify many of these defenses (Gatehouse, 2002). For example, larvae of the tent caterpillars (*Malacosoma* genus) have evolved synchronized feeding with the phenology of their host tree to avoid the physical and chemical defenses of mature foliage, including phytochemical polyphenols such as tannins (Fitzgerald, 1995).

Many inducible plant defenses include proteinase inhibitors (PIs), which are antinutritive proteins that bind tightly to reactive sites of digestive protease, thereby inhibiting protein digestion and often resulting in reduced herbivore growth or starvation (Ryan, 1990). Plant PIs are often effective inhibitors of insect gut proteases *in vitro*, suggesting that many PIs should be correspondingly detrimental to insects if ingested; however, when incorporated into artificial diets or expressed in plant tissue, such PIs may have little or no effect *in vivo* (Jongsma and Bolter, 1997). Inspection of protease activity in the midguts of larvae that ingested PIs demonstrated that insect digestive physiology adapts to the PI. These adaptations include increased total protease activity, which can result from hyperproduction of proteases (De Leo et al., 1998; Markwick et al., 1998), or active protease switching from those that are sensitive to inhibition to those that are resistant (Broadway, 1995; Jongsma et al., 1995). This adaptive switch to PI-resistant protease activity appears to be specifically induced by the PIs rather than a passive response to reduced protein uptake, since the response cannot be mimicked by lower dietary protein or starvation (Bown et al., 1997) and persists even if PIs are added to diets

with hydrolyzed protein (Bown et al., 2004). The increase of PI-resistant protease activity results from induced transcription of the corresponding genes, and may also include repression of genes encoding PI-sensitive proteases (Bown et al., 1997; Mazumdar-Leighton and Broadway, 2001a; Mazumdar-Leighton and Broadway, 2001b; Bown et al., 2004). Sequence comparisons of PI-sensitive and PI-resistant proteases suggest that changes to key amino acids that interact with inhibitors are responsible for resistance (Bown et al., 1997; Lopes et al., 2004; Chougule et al., 2005). This supports the idea of an ongoing evolutionary arms race between insect and plant host.

Most studies of the effects of plant PIs on insect digestive physiology are focused on short-lived herbaceous plants of agronomic importance, and little is known about the defenses in perennials such as trees. Furthermore, protease activity of midguts from forest tree insect herbivores has been reported by only a few studies, such as gypsy moth (*Lymantria dispar*) and western spruce budworm (*Choristoneura occidentalis* (Valaitis, 1995; Valaitis et al., 1999). However, forest insect herbivores pose major challenges to sustainability of both natural and planted forests, and can negatively impact wood quality and quantity. During population outbreaks of forest tent caterpillar (FTC; *Malacosoma disstria*), millions of hectares of trees can be defoliated and repeated episodes of defoliation by FTC larvae during prolonged outbreaks may eventually kill trees (Fitzgerald, 1995).

In *Populus*, the first poplar PI (*win3*) was described by Gordon and co-workers (Bradshaw et al., 1990; Hollick and Gordon, 1993, 1995). Work in this laboratory later showed that two *win3* paralogs from *P. tremuloides* have properties consistent with a role in herbivore defense (Haruta et al., 2001a). Recent genomic projects have identified many additional TIs from poplar that are highly expressed after wounding and herbivory (Chapter 2; Christopher et al., 2004; Ralph et al., 2006a). Together they form a rapidly evolving and genetically diverse multigene family (Ingvarsson, 2005; Talyzina and Ingvarsson, 2006). We recently characterized five representative members of this family that likely have roles in herbivore defense, and showed that they are functional inhibitors, with distinct inhibitor profiles and substrate preferences (Chapter 5). Moreover, at least one of these TIs (TI3) was an inhibitor of midgut proteases from *Mamestra configurata*, an insect pest of crucifers. Here we study the effects of the five recombinant poplar TIs

against FTC, a pest of aspen and poplar, with *in vitro* and *in vivo* assays. We show that all five poplar TIs inhibit FTC protease activity with various potencies, and that poplar TI3 had the strongest inhibitory activity, comparable with STI potency. In feeding trials, both TI3 and STI had detrimental effects on FTC larval growth. Furthermore, larvae which ingested TI3 appear to secrete higher levels of digestive proteases to counteract the effects of TI3 inhibition, while STI ingestion causes reduced protease activity but with an accompanying adaptive switch to proteases that are resistant to STI.

6.2 Materials and Methods

In vitro protease and protease inhibitor assays

FTC midguts were extracted into insect Ringer's solution, clarified by centrifugation and protein quantified by the method of Bradford (1976). Protease activity of midgut extracts was determined by incubating a standard quantity of extract in assay buffer (0.1 M Tris-HCl for pH 7 - 9; 0.1 M glycine-NaOH for pH 9.5 - 13) with 1% azocasein (Sigma) for 6 h at 23 °C, after which the reaction was stopped by addition of trichloroacetic acid. The A_{450} was measured and used to calculate protease activity. Specific activity was defined as units of protease activity per minute per mg protein, and one unit was defined as the quantity of protease activity required for hydrolysis of azocasein to increase absorbance at 450 nm by one unit. All midgut protease assays were performed in triplicate and results are the mean of three assays. To determine the pH optima for FTC midgut proteases, total protease activity was quantified in the buffers 0.1 M Tris-HCl (pH 7 - 9) and 0.1 M glycine-NaOH (pH 9.5 - 13). Assays were read in 96-well plates with a Tecan Sunrise plate reader (Salzburg, Austria).

To characterize the composition of FTC midgut protease activity, chemical protease inhibitors were pre-incubated with midgut extract and the remaining protease activity was quantified by azocasein hydrolysis as described above. Serine protease activity was assessed by inhibition with PMSF (phenylmethylsulfonyl fluoride; Sigma), trypsin activity with TLCK (N_{α}-tosyl-L-lysyl-chloromethane hydrochloride; Sigma), chymotrypsin activity with TPCK (N-*p*-tosyl-L-phenylalanine chloromethyl ketone; Sigma), cysteine protease activity with iodoacetamide (Sigma), aspartic protease activity

with pepstatin (Sigma) and metalloprotease activity with EDTA (ethylenediamine tetraacetic acid).

Inhibition of midgut protease activity by poplar TIs and soybean STI was determined by pre-incubating increasing amounts of TI with a standard quantity of midgut extract (normalized by protein content), and residual protease activity was quantified by azocasein hydrolysis as described above. Soybean TI (STI; Sigma) was used as a positive control for all assays. The TI concentrations required to inhibit 50% protease activity (IC_{50}) were determined from the linear portion of the plot of residual protease activity against TI protein (μg).

Heterologous expression and purification of recombinant TI proteins

TI3 was expressed and purified as described previously (Chapter 5). Briefly, recombinant TI proteins were produced and isolated from *E. coli* inclusion bodies, and purified on a nickel affinity resin (Ni-NTA Agarose, Qiagen) along a pH gradient under denaturing conditions (8 M urea). Denatured proteins were re-folded by dialyzing against 50 mM Tris-HCl buffer (pH 8.0) at 4°C. Protein concentrations were determined spectrophotometrically (A_{280}) and quality was verified on Coomassie-stained SDS-PAGE gels. For *in vitro* assays, STI was dissolved in ddH_2O and dialyzed against 50 mM Tris-HCl buffer (pH 8.0), so that buffers for poplar TIs and STI were identical. For feeding assays with TI3, more than 200 mg TI3 was produced and purified as described above. Quality was verified by Coomassie-stained SDS-PAGE and *in vitro* trypsin inhibitor assays.

Feeding trials

Feeding trials were carried out by Dr. Emma Despland (Concordia University), with recombinant protein produced at the University of Victoria, using previously described methods (Despland and Noseworthy, 2006). FTC larvae were reared on artificial diets of two dietary ratios of protein and carbohydrate (Despland and Noseworthy, 2006). The optimal protein diet consisted of 21% protein and 21% digestible carbohydrate (by dry mass; 21:21) and the reduced protein diet consisted of 14% protein and 28% carbohydrate (14:28). TI3 and STI were incorporated into diets at

0.04% and 0.2% TI protein (wet weight). These concentrations are equivalent to approx. 20 μM and 100 μM, respectively, which is within the range of reported TI concentrations of induced leaves (Jongsma and Bolter, 1997; Haruta et al., 2001a). Feeding experiments were continued until larvae development to fourth or fifth instars, at which point midguts were removed from healthy larvae and frozen in liquid nitrogen for analysis of protease activity. Midgut protease assays were carried out to compare protease activity of larvae fed diets with and without TI3 to determine the effects of TI on digestive physiology. For analysis of midgut protease activity of FTC larvae from the TI3 feeding experiments, larvae fed control 21:21 diets without TI3 had high levels of mortality and could therefore not be analyzed. However, since the midgut protease activities are similar for larvae from different feeding experiments, we were able to use control midguts from previous feeding experiments.

6.3 Results

Characterization of FTC larval midgut protease activity

Prior to examining the effects of plant PIs, we characterized protease activity of whole midguts extracted from FTC larvae. Using azocasein as a general protease substrate, we measured protease activity between pH 7.0 and 13.0, since lepidopteran digestive proteases have alkaline pH optima and whole midguts extracts are heterogeneous and can contain proteases with different pH optima (Terra and Ferreira, 1994; Hegedus et al., 2003). Maximum protease activity was measured between pH 9.5 and 13.0, with an optimum at pH 11.5 (Fig. 6-1). We further characterized the protease activity using specific inhibitors to determine the contribution of different protease classes to overall activity. For this experiment, we measured activity from pH 7.5 to 12.5 to determine if the contribution of different proteases changes with pH. Our results show that serine proteases were responsible for virtually all total protease activity of FTC midguts at all pH units tested, since PMSF inhibited more than 90% of protease activity from pH 7.5 - 10.5 (Fig. 6-2 and Table 6-1). In addition, the majority of the serine protease activity appears to be trypsin-like, as the trypsin-specific inhibitor TLCK inhibited more than 65% activity from pH 9.5 - 11.5, and as much as 80% inhibition at pH 11.5. A smaller chymotrypsin-like component was also present, since the

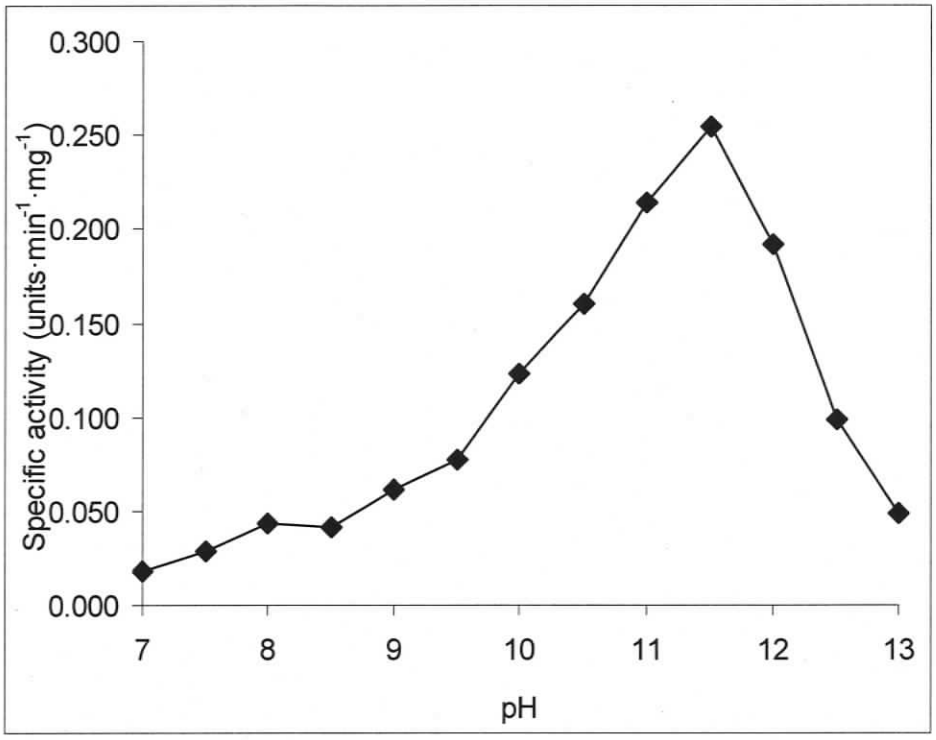


Figure 6-1. Effect of pH on *Malacosoma disstria* larval midgut protease activity. Midgut extracts were assayed for total protease activity using azocasein as substrate with the assay buffers Tris-HCl (pH 7.0 - 9.0) and glycine-NaOH (pH 9.5 - 13.0).

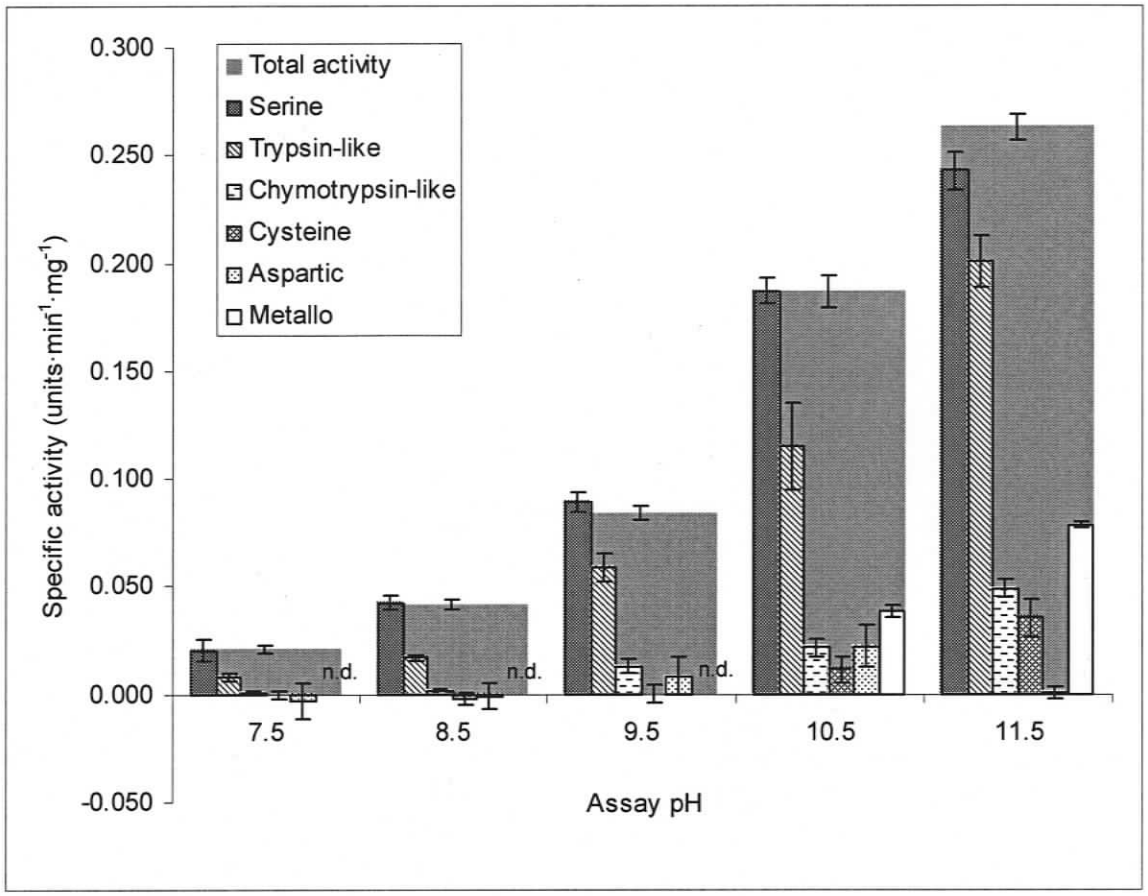


Figure 6-2. Characterization of protease activities in *Malacosoma disstria* larval midgut extracts.

Midgut extracts were assayed for protease activity with azocasein as a substrate. Effect of pH was determined by varying the buffer pH using Tris-HCl (pH 7.5, 8.5) and glycine-NaOH (pH 9.5 - 11.5). Contribution of serine-type, trypsin-like, chymotrypsin-like, cysteine-type, aspartic-type and metalloprotease activities to total activity were determined by addition of the protease inhibitors PMSF (25 mM), TLCK (10 mM), TPCK (1 mM), iodoacetamide (1 mM), pepstatin A (10 mM) and EDTA (50 mM), respectively. Each assay was replicated at least three times and error bars show standard error.

Table 6-1. Effect of protease inhibitors on total proteolytic activity of midgut extracts from forest tent caterpillar (*Malacosoma disstria*)[a].

Protease inhibitor and class			Inhibition (%) ± SE				
			pH 7.5	pH 8.5	pH 9.5	pH 10.5	pH 11.5
Serine protease	PMSF	1 mM	48.5 ± 18.7	39.4 ± 6.4	30.1 ± 5.5	15.3 ± 3.1	11.1 ± 1.5
		10 mM	80.9 ± 13.6	80.8 ± 5.0	96.2 ± 2.6	55.5 ± 9.6	50.2 ± 7.6
		25 mM	95.8 ± 6.8	100.0 ± 5.9	100.9 ± 0.4	92.8 ± 1.2	87.6 ± 2.0
	TLCK	0.1 mM	18.1 ± 1.0	20.2 ± 0.5	24.6 ± 3.7	33.1 ± 2.4	38.7 ± 3.6
		1 mM	24.5 ± 4.8	16.8 ± 9.6	35.3 ± 3.7	47.0 ± 2.6	63.4 ± 1.0
		10 mM	41.8 ± 8.5	44.2 ± 2.2	76.9 ± 6.9	67.2 ± 10.6	78.5 ± 3.2
	TPCK	0.1 mM	-20.8 ± 13.8	-1.9 ± 1.9	6.7 ± 0.9	4.3 ± 1.1	7.3 ± 0.4
		1 mM	5.4 ± 6.0	5.8 ± 1.7	17.2 ± 3.2	13.3 ± 1.9	19.8 ± 0.7
Cysteine protease	IAA	0.1 mM	1.4 ± 3.7	-7.5 ± 4.1	2.8 ± 1.6	-1.5 ± 2.1	0.4 ± 1.4
		1 mM	-1.1 ± 7.5	-4.5 ± 6.8	0.7 ± 5.0	7.3 ± 2.6	14.6 ± 2.6
Aspartic proteases	Pepstatin A	1 µM	-29.1 ± 40.6	-8.0 ± 10.3	6.3 ± 3.9	8.4 ± 1.4	2.1 ± 3.1
		10 µM	-24.1 ± 29.2	-9.0 ± 15.8	8.8 ± 3.4	10.5 ± 3.1	0.2 ± 3.8
Metallo-proteases	EDTA	5 mM	-9.9 ± 11.4	-20.6 ± 13.4	2.2 ± 5.1	3.8 ± 2.9	10.2 ± 1.0
		50 mM	n.d.	n.d.	n.d.	19.0 ± 1.8	29.0 ± 0.6

[a] Total proteolytic of crude extracts was assayed by azocasein hydrolysis at the pH shown with inhibitor added to the concentration shown and percent inhibition is presented as the percentage of activity without inhibitor added. Standard error is calculated from three replicate assays. n.d. = not determined

chymotrypsin-specific inhibitor TPCK inhibited 13 - 20% activity at pH 9.5 - 11.5. Cysteine and metalloproteases may also contribute to overall protease activity, since iodoacetamide inhibits approx. 15% of activity at pH 11.5 and EDTA inhibits 19 - 29% of activity at pH 10.5 - 11.5, respectively (Fig. 6-2 and Table 6-1). However, the effects of iodoacetamide and EDTA may be non-specific, so activity should be verified with other inhibitors and specific substrates of cysteine and metalloproteases (see Discussion). Pepstatin did not appreciably reduce protease activity (only 10% maximum inhibition at pH 10.5), indicating that aspartic proteases are not significantly active in FTC midguts.

Effect of plant PIs on protease activity in vitro

We previously showed that five poplar TI proteins are active protease inhibitors and that they have distinct functional profiles (Chapter 5). We further tested these poplar TIs, as well as soybean TI (STI), for inhibition of FTC midgut protease activity using azocasein as a protease substrate. All of the poplar TIs tested inhibited midgut protease activity (Fig. 6-3), although TI4, TI5 and TI6 were much weaker inhibitors than TI2 and TI3. To quantify the inhibitor strength of the TIs, we calculated maximum inhibition of the TIs, as well as the TI concentration at which 50% of protease activity was inhibited (IC_{50}; Fig. 6-3C). Since the midguts contain a heterogeneous mixture of proteases, the maximum inhibition represents the proportion of protease activity sensitive to each TI. The IC_{50} provides a measure of the affinity of the TI and protease; smaller values translate to higher affinities and stronger inhibition. TI4, TI5 and TI6 had higher IC_{50} values and only inhibited a maximum of 10 - 20% of midgut protease activity. TI2 was a stronger inhibitor, with an IC_{50} that was an order of magnitude lower than TI4, TI5 and TI6. Furthermore, TI2 inhibited at least 40% of the protease activity. TI3 and STI were by far the most potent inhibitors of FTC midgut proteases (Fig. 6-3). TI3 was less potent than STI based on IC_{50} values, but both TIs inhibited approx. 70% of protease activity. Therefore, of the poplar TIs tested, TI3 is the strongest inhibitor of FTC protease activity *in vitro*, and a good candidate for further analysis of the *in vivo* effects of poplar TIs on FTC larvae.

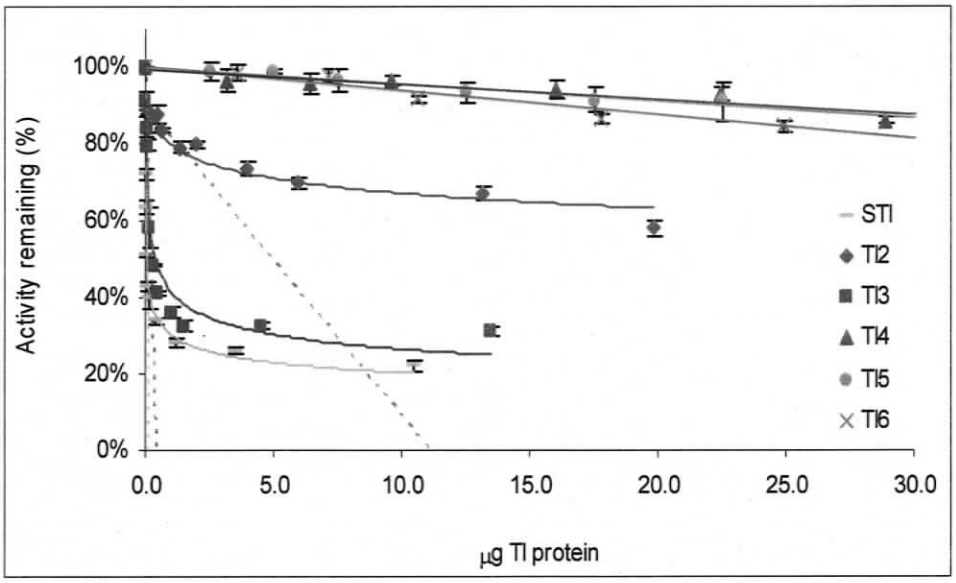
Figure 6-3. Effect of poplar and soybean plant protease inhibitors against larval midgut proteases from *Malacosoma disstria*.

A. Titration of poplar TIs TI2 (blue diamond), TI3 (red square), TI4 (purple triangle), TI5 (orange circle) and TI6 (green 'x'), as well as soybean TI (STI; yellow dash) against larval midgut extracts.

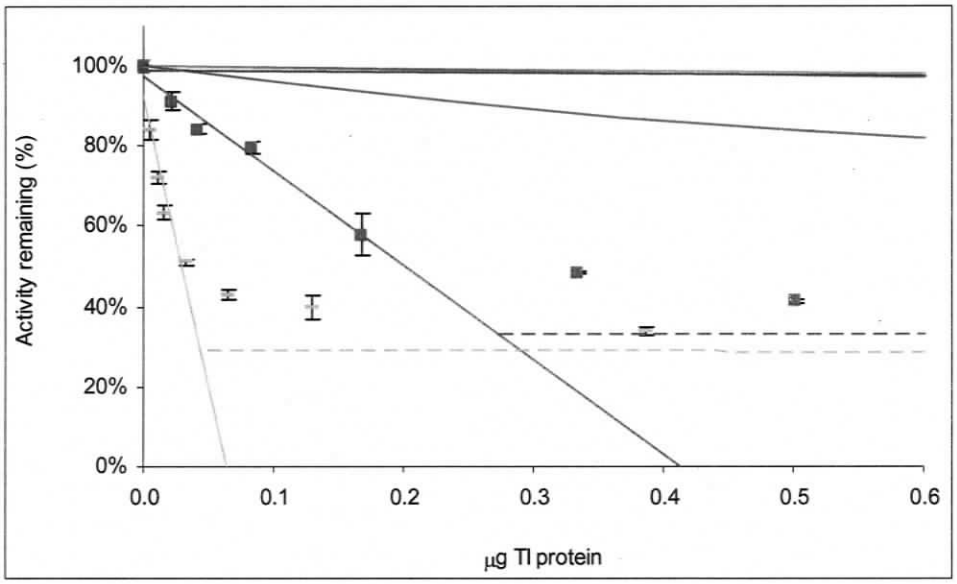
B. The same data are re-plotted using a smaller-scale abscissa and with hidden points for TI2, TI4, TI5 and TI6 to better visualize TI3 and STI protease inhibition.

C. Maximum inhibition (%) was determined and linear regression used to calculate 50% inhibitory concentration (IC_{50}) to further describe inhibition by these plant protease inhibitors. Error bars are standard error.

A



B



C

	TI2	TI3	TI4	TI5	TI6	STI
IC_{50} (µM)	0.764	0.0763	8.78	5.32	8.32	0.0129
Maximum inhibition (%)	≥ 41.92	67.97	≥ 13.95	≥ 8.88	≥ 18.09	74.42

Figure 6-3.

In vivo effects of ingested plant PIs

Considering their strengths as inhibitors of proteolytic activity *in vitro*, we speculated that TI3 and STI should also have strong effects *in vivo*. To assess the effectsof TI3 and STI on FTC growth and development, larvae were reared on artificial diets supplemented with 0.04% and 0.2% TI3 and STI. Since protein concentration can influence the effects of PIs (Burgess et al., 1991; Markwick et al., 1995), two levels of dietary protein were tested with TI addition. Here, we define the 21:21 diet as optimal protein, which is optimal for FTC growth and development, and the 14:28 diet as reduced protein, which causes reduced growth and delayed development (Despland and Noseworthy, 2006). Experiments incorporating TI3 into optimal protein diets showed that TI3 reduced larval growth, since larval weights decreased substantially after feeding on both 0.04% and 0.2% TI3 (Fig. 6-4A). By contrast, when TI3 was added to reduced protein diets, it showed no effect. However, larvae grew substantially slower on reduced protein diets, such that growth on 14:28 diets without TI3 was lower than larvae fed 21:21 diets with TI3. STI feeding experiments revealed that STI also reduced larval growth, with substantially reduced larval weights for larvae fed optimal protein diets incorporating either 0.04% or 0.2% STI (Fig. 6-4B). Larvae again grew substantially slower on reduced protein diets, and addition of STI did not further reduce growth. Interestingly, some larvae grew normally on 0.2% STI; these larvae were separated from smaller larvae for later analyses of digestive physiology (see below). It appears that any negative effect caused by TI3 or STI in the reduced protein diet is masked by the negative effects of the lower dietary protein (see Discussion). Overall, our results clearly demonstrate that poplar TI3, as well as STI, negatively effect FTC larval growth, and are therefore effective defense proteins.

Effects of ingested plant PIs on digestive physiology

To assess how ingestion of TI3 and STI affect FTC digestive physiology and consequent larval growth, protease activity was determined from midguts of larvae following feeding trials at both levels of dietary protein. Total protease activity was similar when larvae were fed 21:21 and 14:28 diets (0% TI3, Fig. 6-5A). When larvae were exposed to diets with TI3, total protease activity increased (Fig. 6-5A). This

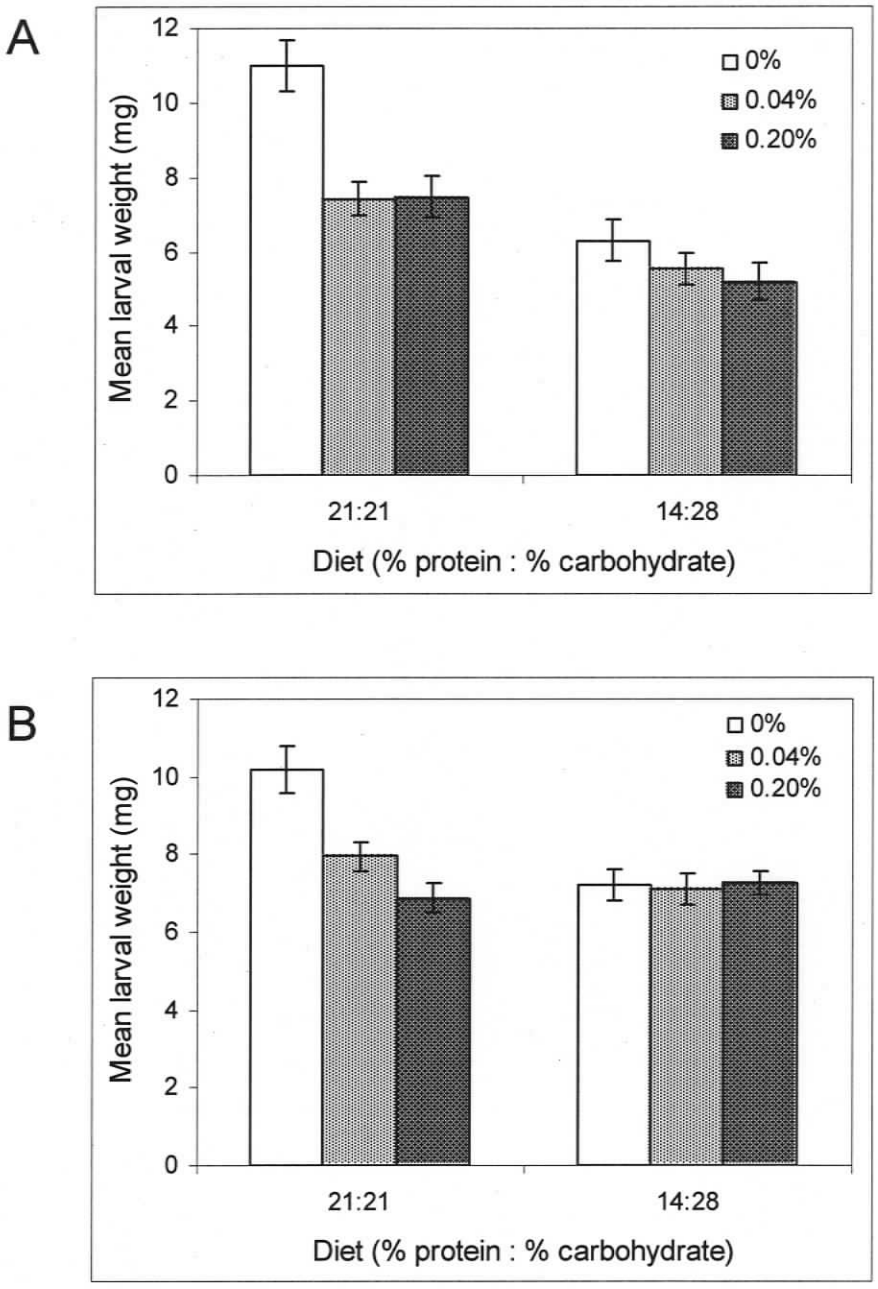


Figure 6-4. Effect of TI3 and STI addition to artificial diets on FTC larval growth.

Mean fresh weight of second instar larvae fed optimal protein (21:21) and reduced protein (14:28) artificial diets supplemented with 0%, 0.04% and 0.2% poplar TI3 (**A**) or soybean TI (**B**).

[Experiment carried out by Dr. Emma Despland, Concordia University.]

Figure 6-5. Effects of ingested TIs on total protease activity of midgut extracts from *Malacosoma disstria* larvae.

Rates of hydrolysis of azocasein at pH 11.5 were used to determine specific protease activity of larvae fed optimal protein (21% protein:21% carbohydrate) and reduced protein (14% protein:28% carbohydrate) diets incorporating 0.04% and 0.2% poplar TI3 (**A**) or optimal protein diets incorporating 0.2% STI (**B**). For diet incorporating 0.2% STI, larvae with reduced growth (small, S) and normal growth (large, L) were analyzed separately. Control larvae from the TI3 feeding experiment suffered unusually high mortality, and therefore the equivalent 0% STI controls were used for these comparisons (asterisk/italics; A). White bars, high protein diet; hatched bars, low protein diet. Data values are shown above each column; error bars are standard error of three replicates.

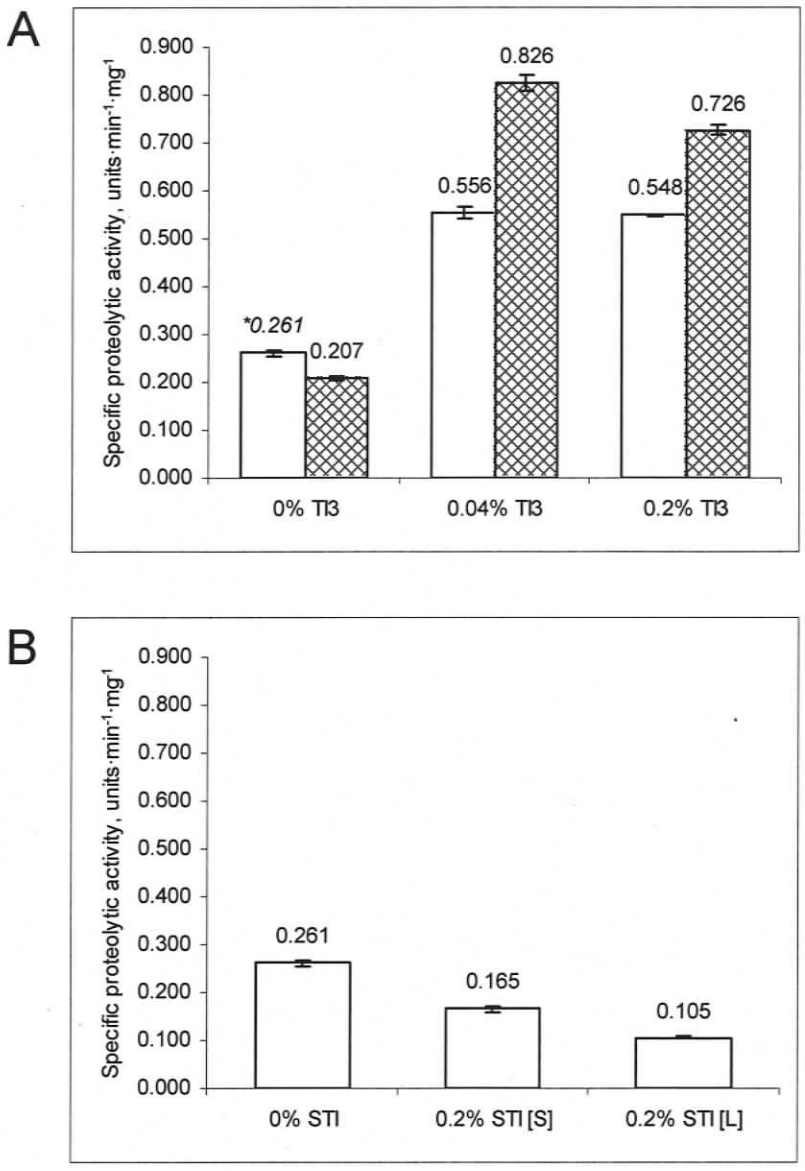

A
Specific proteolytic activity, units·min-1·mg-1
0.900
0.800
0.700
0.600
0.500
0.400
0.300
0.200
0.100
0.000
*0.261
0.207
0.556
0.826
0.548
0.726
0% TI3
0.04% TI3
0.2% TI3
B
Specific proteolytic activity, units·min-1·mg-1
0.900
0.800
0.700
0.600
0.500
0.400
0.300
0.200
0.100
0.000
0.261
0.165
0.105
0% STI
0.2% STI [S]
0.2% STI [L]

Figure 6-5.

increase was not TI3 dose-dependent and was measured for both dietary protein levels, with a greater increase when TI3 was added to low protein diets. By contrast, when STI was incorporated in diets (0.2%) total protease activity decreased 37% for smaller larvae exhibiting reduced growth (Fig. 6-5B, 0.2% STI [S]). Interestingly, those larvae that were unaffected by STI and grew normally showed a more pronounced 60% decrease of activity (Fig. 6-5B, 0.2% STI [L]).

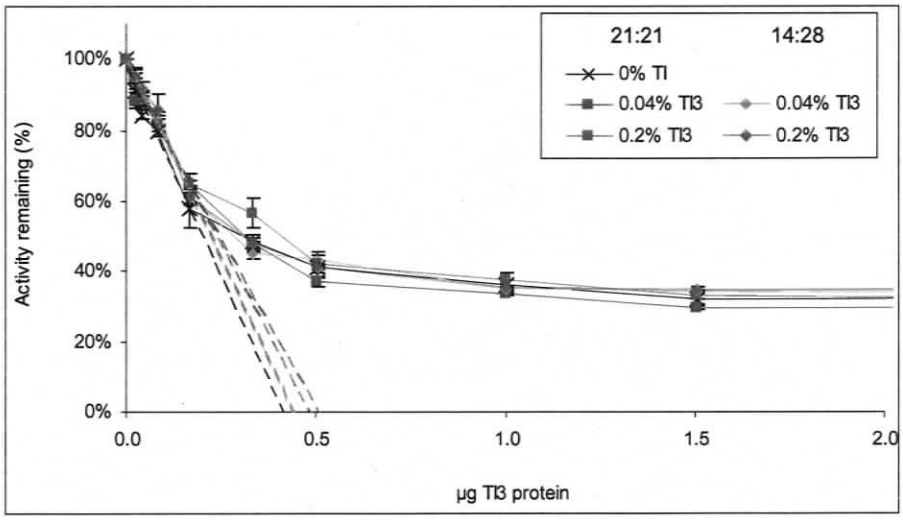
To determine whether TI3 and STI ingestion lead to the production of TI-resistant proteases in FTC larvae, we measured TI3 and STI inhibition of FTC midgut protease activities from the same feeding experiments (i.e. larvae that had been exposed to TI3 and STI for several days). For larvae fed diets with TI3, *in vitro* assays of midguts proteases with both TI3 and STI demonstrated that IC_{50} values and maximum protease inhibition were unaltered by both dietary protein level and TI3 addition (Fig. 6-6 and Supplemental Fig. 6-1). This indicates that ingestion of TI3 did not cause a change in the relative sensitivity of gut proteases to TI3 or STI. For larvae fed optimal protein diets incorporating STI, we analyzed activity from small (reduced growth) and large (growth unaffected) larvae separately. STI ingestion did not change IC_{50} values, indicating that the affinity of STI-protease interactions was not affected (Fig. 6-7A,C). However, maximum protease inhibition was noticeably lower for small larvae (Fig. 6-7B,C), demonstrating that midguts contained a higher proportion of STI-resistant proteases resulting in gut protease activity that was more resistant to STI. We found similar results for these midgut extracts when we measured *in vitro* inhibition by TI3; IC_{50} values were similar for all extracts and maximum protease inhibition was lower for small larvae (Fig. 6-7C and Supplemental Fig. 6-2). Thus, ingestion of STI appeared to trigger an increase of protease activity that was resistant to both STI and TI3.

We next examined the contribution of different protease classes to the overall midgut protease activity of larvae from TI3 and STI feeding experiments to determine whether broad changes in protease class composition occurred after TI ingestion. We estimated the specific activity of each protease class using specific inhibitors from pH 9.5 to 11.5 (as described before). The effects of TI3 and STI ingestion on midgut protease activity were consistent with our previous results at pH 11.5 (Fig. 6-8); TI3 ingestion increased total activity while STI ingestion reduced total protease activity, irrespective of

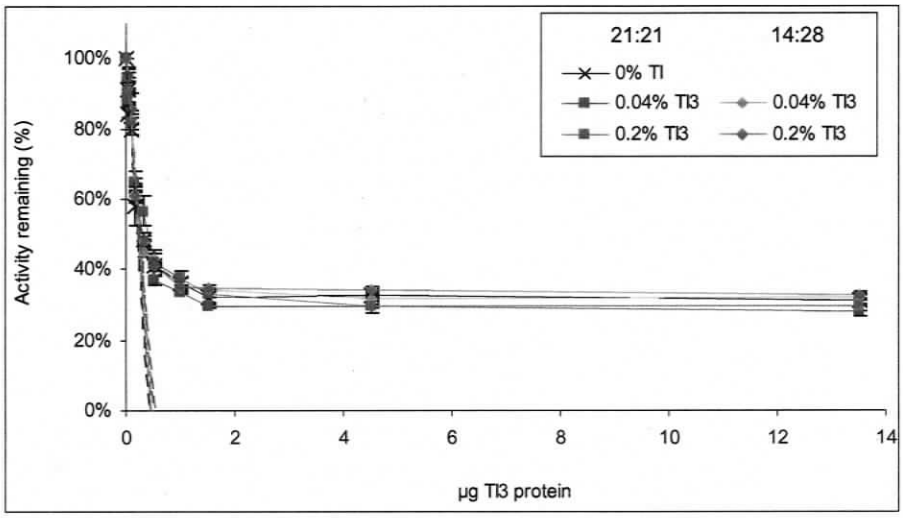
Figure 6-6. Effect of ingestion of poplar TI3 on levels of TI3-resistant protease activity in midguts from *Malacosoma disstria* larvae from TI3 feeding experiments.

Larvae were fed optimal protein (21:21) and reduced protein (14:28) diets incorporating 0.04% or 0.2% TI3, and TI-resistant protease activity was determined by titration of TI3 and soybean TI (STI) with midgut extracts, followed by azocasein hydrolysis at pH 11.5. TI3 inhibition is plotted on small- (**A**) and large-scale abscissae (**B**) to visualize the similar inhibition profiles and maxima of proteases from larval fed diets with and without TI3. Error bars are standard error of three technical replicates. Maximum protease inhibition and the half-concentration of TI required to achieve maximum inhibition were determined from these *in vitro* assays for titration of both TI3 and soybean TI (**C**).

A



B



C

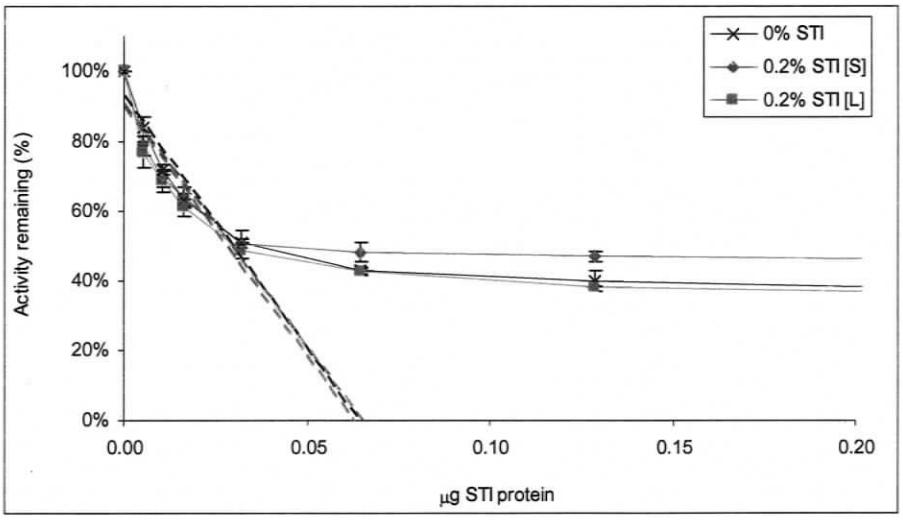
		IC_{50} (nM)		Maximum inhibition (%)	
In vitro assay with:		TI3	STI	TI3	STI
21:21	0% TI3	76.3	12.9	68.0	74.4
21:21	0.04% TI3	95.6	13.0	70.7	74.5
21:21	0.2% TI3	94.5	11.3	69.0	75.9
14:28	0.04% TI3	84.4	13.1	67.3	69.2
14:28	0.2% TI3	85.5	12.5	66.1	71.8

Figure 6-6.

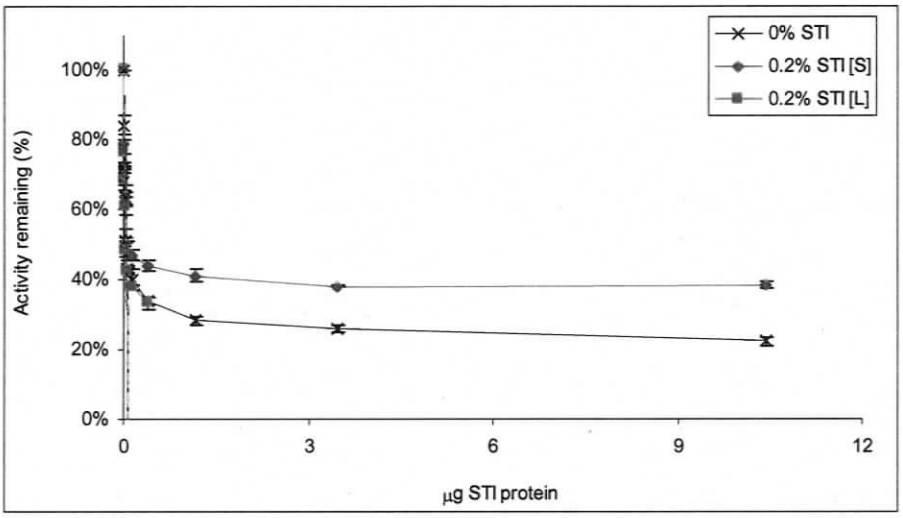
Figure 6-7. Effect of ingestion of soybean TI (STI) on levels of STI-resistant protease activity in midguts from *Malacosoma disstria* larvae from STI feeding experiments.

Larvae were fed optimal protein diet incorporating 0.2% STI, and TI-resistant protease activity was determined by titration of STI and poplar TI3 with midgut extracts, followed by azocasein hydrolysis at pH 11.5. STI inhibition is plotted on small- (**A**) and large-scale abscissae (**B**) to visualize the similar inhibition profiles yet different inhibition maxima of proteases from larvae fed diet with 0.2% STI with normal growth (large, L) and reduced growth (small, S) compared with larvae fed diet without STI. Error bars are standard error of three technical replicates. Maximum protease inhibition and the half-concentration of TI required to achieve maximum inhibition were determined from these *in vitro* assays for titration of both STI and poplar TI3 (**C**).

A



B



C

	IC_{50} (nM)		Maximum inhibition (%)	
In vitro assay with:	STI	TI3	STI	TI3
0% STI	12.9	76.3	74.4	68.0
0.2% STI [S]	9.32	82.8	60.8	51.9
0.2% STI [L]	9.87	98.9	≥ 66.7	67.9

Figure 6-7.

Figure 6-8. Effect of ingested poplar TI3 and soybean TI (STI) on activity of serine-type proteases in midguts from *Malacosoma disstria* larvae.

Protease activities of larvae fed artificial diets supplemented with no TI, 0.04% and 0.2% poplar TI3, and 0.2% STI were determined by azocasein hydrolysis for the pH range 9.5 - 12.5. Total serine protease activity (**A**), trypsin-like activity (**B**) and chymotrypsin-like activity (**C**) were determined by addition of the protease inhibitors PMSF (25 mM), TLCK (10 mM) and TPCK (1 mM), respectively. Error bars are standard error of three replicates. Total proteolytic activity measured in the absence of these inhibitors is shown as a grey shadow.

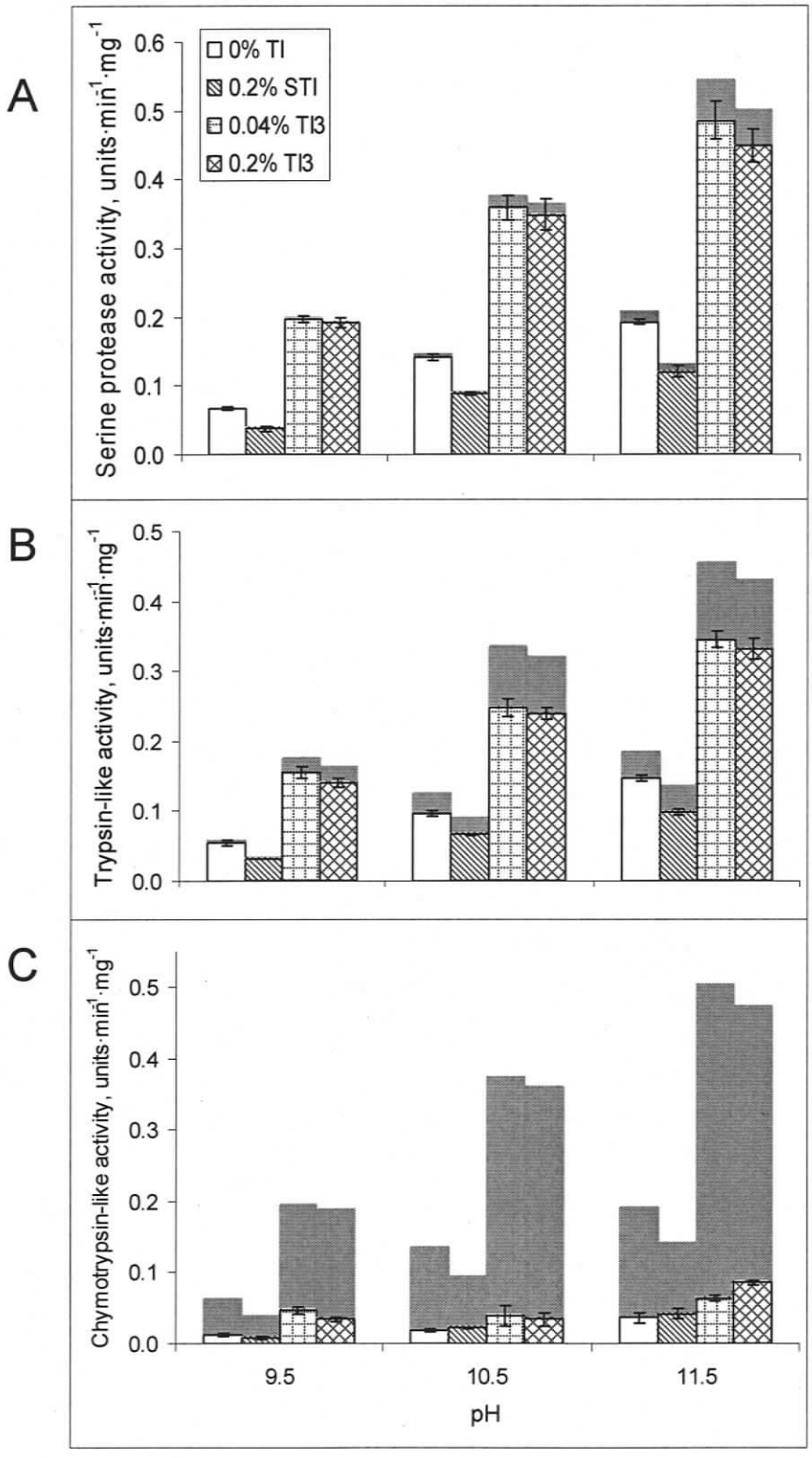

A
B
C
0% TI
0.2% STI
0.04% TI3
0.2% TI3
Serine protease activity, units·min-1·mg-1
Trypsin-like activity, units·min-1·mg-1
Chymotrypsin-like activity, units·min-1·mg-1
0.0
0.1
0.2
0.3
0.4
0.5
0.6
9.5
10.5
11.5
pH

Figure 6-8.

pH (compare Fig. 6-5 and 6-8). Despite the overall changes in protease activity after TI ingestion, there was no appreciable change in the contribution of different protease classes. Total protease activity of midguts from larvae fed diets with STI and TI3 still consisted primarily of serine protease activity, most of which was trypsin-like with a minor chymotrypsin-like component (Fig. 6-8 and Supplemental Table 6-1). However, our results suggest modest changes in the contribution of chymotrypsin-like activity after ingestion of STI and TI3. Relative chymotrypsin-like activity increased slightly for larvae fed STI, while there was a small decrease in chymotrypsin-like activity larvae after TI3 ingestion (Supplemental Table 6-1). Our results also suggest possible minor changes of metalloprotease-like activity after TI3 ingestion, since EDTA inhibition was decreased. However, the potential changes in protease activity reflected by changes of TPCK and EDTA inhibition are minor, and thus the contribution of different protease classes to overall activity did not change appreciably after TI consumption.

6.4 Discussion

This study examined the interaction of poplar TI proteins with FTC midgut proteases to further establish the roles of TIs in herbivore defense. Analysis of FTC midgut protein extracts showed that protease activity consists primarily of serine-type proteases, and especially trypsin-like enzymes. All five poplar TIs tested exhibited at least some inhibition of protease activity, with poplar TI3 and soybean STI the strongest inhibitors. TI3 and STI were therefore used in feeding experiments by incorporating the TIs in optimal and reduced protein diets. Both TI3 and STI reduced larval growth when incorporated optimal protein diet. Analysis of larval midgut extracts from these feeding experiments revealed different adaptations of midgut digestive physiology to TI3 and STI. TI3 ingestion caused an increase of midgut protease activity but not increased secretion of TI3-resistant proteases. This may reflect a hyperproduction of digestive proteases to compensate for TI3 inhibition. By contrast, STI ingestion reduced midgut protease activity but increased STI-resistant protease activity, consistent with adaptation to STI by protease switching.

Characterization of FTC larval midgut protease activity

This is the first study to characterize digestive proteolytic activity of FTC, which is optimal at pH 11.5 (Fig. 6-1) and consistent with the physiological pH of FTC midguts that have been measured as high as 10.4 (Heimpel, 1955). Consistent with an alkaline pH optimum, total protease activity of FTC midguts is almost entirely due to serine-type, and mostly trypsin-like proteases (Fig. 6-2 and Table 6-1). The protease activity of most lepidopterans also consists primarily of serine-type proteases (Christeller et al., 1992; Terra and Ferreira, 1994). We note that our results approximate the contributions of different protease classes, since we used one inhibitor to assess the contribution of each protease class to total activity. Some other studies have shown that unlike mammalian chymotrypsins, lepidopteran chymotrypsin-like proteases may be resistant to inhibition by TPCK (Johnston et al., 1995; Lee and Anstee, 1995), and thus our results may underestimate chymotrypsin-like activity of FTC midguts. However, since some protease activity is inhibited by TPCK, chymotrypsin-like proteases clearly contribute to at least a portion of total FTC gut protease activity. Overall, our results indicate that the predominant FTC protease activity is due to trypsin-like proteases. Additional analyses with specific substrates and additional inhibitors will help better define the types of proteases found in FTC.

Poplar TIs as well as STI all inhibited proteolytic activity in FTC midgut extracts *in vitro* (Fig. 6-3). We previously showed that the poplar TIs are inhibitors of trypsin, chymotrypsin or both (Chapter 5). STI is a well-characterized inhibitor of trypsins and chymotrypsins from a wide range of lepidopteran families (Terra and Ferreira, 1994). It is interesting that all five poplar TIs inhibit FTC protease activity, since only TI3 inhibited midgut extracts from *Mamestra configurata* (Chapter 5). However, poplar TIs inhibit FTC midgut proteases with variable effectiveness, and TI3 is also by far the most effective inhibitor of FTC proteases (Fig. 6-3C). These results are consistent with the functional diversity that we previously showed for inhibition of mammalian proteases (Chapter 5). In addition, the variable maximum inhibitions of FTC protease activity suggest that FTC midguts contain multiple proteases that are differentially inhibited by the TIs. It would be interesting to test combinations of poplar TIs to determine if they inhibit distinct or common proteases. At least one FTC protease is resistant to TI

inhibition and contributes as much as 25% of total protease activity, since STI and TI3 never inhibited more than 68-74% of total activity. Similar proportions of STI-resistant proteases have been reported for other lepidopteran midguts (Hegedus et al., 2003; Bown et al., 2004).

Effects of ingested plant PIs on larval growth and digestive physiology

As predicted from their effective inhibition of midgut proteases *in vitro*, TI3 and STI both reduce FTC larval growth when ingested. At 0.04%, the STI and TI3 concentrations are within the range of PI levels found in herbivore-induced leaves (Jongsma and Bolter, 1997), including levels of TI2 proteins in wounded aspen leaves (Haruta et al., 2001a). Interestingly, while TI3 and STI reduce larval growth when incorporated in optimal protein diets, they have no effect in reduced protein diets. Dietary protein, including protein concentration and amino acid composition, has been shown to modulate the severity of the effects caused by PIs on insects (Jongsma and Bolter, 1997). As in our experiments, PIs (including STI) reduced *Cydia pomonella* growth more effectively when dietary protein was not limiting (Markwick et al., 1995). By contrast, other studies have found that PIs are more effective in low protein diets and suggested that the lower dietary protein exacerbates reduced protein digestion caused by PIs (Broadway and Duffey, 1986; Burgess et al., 1991). It is possible that we do not observe TI growth reduction for reduced protein diets because of the overall growth reduction associated with the reduced protein diet itself. Growth reduction was previously observed for FTC fed the same 14:28 diet compared with 21:21 diet (Despland and Noseworthy, 2006).

Digestive physiologies of larvae fed diets incorporating TI3 and STI differ from those fed control diets, demonstrating an adaptive response of FTC to ingestion of TIs, and confirming the impact of TIs on insect digestive physiology. However, the responses are different for TI3 and STI. Larvae fed diets with TI3 have higher levels of total protease activity (Fig. 6-5A), but there is no change in sensitivity to TI3 or STI *in vitro* (Fig. 6-6) and no change in the proportion of trypsin- and chymotrypsin-like activities (Fig. 6-8). Thus, our results suggest that TI3 ingestion causes hyperproduction of midgut proteases already present in the midgut. This was previously observed for *Epiphyas*

postvittana and *Planotortrix octo* larvae fed diets with potato inhibitor 1 (POT1; a serine PI), which reduced growth of both larval species and resulted in increased trypsin and chymotrypsin activity with no change in sensitivity to POT1 (Markwick et al., 1998). This hyperproduction of proteases, much of which is TI-sensitive, may contribute to growth suppression by depleting essential amino acids used for protease production (Broadway and Duffey, 1986). However, there is no FTC growth reduction associated with TI3 in reduced protein diets, which would contradict the hyperproduction/depletion hypothesis; these larvae have even higher levels of protease activity (Fig. 6-5A) and should therefore exhibit even greater reduced growth. However, by virtue of increasing total levels of gut protease activity, larvae on 14:28 diets with TI3 will have 50 - 75% more specific protease activity that is TI-resistant than larvae on 21:21 diets with TI3 (data not shown). Perhaps this higher level of TI-resistant activity allows FTC larvae to overcome the effects of TI3.

In contrast to the changes caused by TI3 ingestion, larvae reared on diets with STI had reduced levels of total protease activity (Fig. 6-5B). It is possible that this decrease in protease activity measured *in vitro* is due to inhibition by the ingested STI, since we were only able to measure the activity of free proteases. It has been previously shown that PI-bound enzymes contribute to a large fraction of midgut proteases from larvae fed leaves overexpressing a PI (Jongsma et al., 1995). The smaller larvae exhibiting reduced growth on STI-supplemented diet have approx. 50% more TI-resistant protease activity than larvae fed control diets (Fig. 6-7), suggesting an adaptive switch to TI-resistant proteases. While many insect species employ adaptive protease switching as a mechanism to overcome the detrimental effects of dietary PIs (Jongsma and Bolter, 1997), there are few examples of larvae that are negatively affected despite increased PI-resistant protease activity (Broadway, 1995; Montesdeoca et al., 2005). It is possible that higher concentrations of STI could further increase STI-resistant protease activity and allow FTC larvae to overcome the growth reduction, since increased activity of PI-resistant proteases is dependent on STI dosage for several lepidopteran species (Broadway, 1997). While the 0.2% TI concentration is biologically relevant in terms of leaf concentrations, it is lower than many other studies that demonstrate insect adaptation to plant PIs (Jongsma and Bolter, 1997). However, we found no evidence of a dose-dependent response for STI.

It is as yet unclear why some larvae grew normally on STI diets, and more surprising that these larvae have even further reduced levels of protease activity than the growth-reduced larvae reared on STI diets (Fig. 6-5). More detailed analyses of digestive physiology of these larvae may clarify these results.

It is interesting that larvae fed TI3 or STI have contrasting digestive responses; TI3 causes hyperproduction of overall protease activity, while STI generally reduces protease activity but also elicits an increase of resistant protease activity. Although the TI-resistant protease activity from FTC midguts is resistant to both STI and TI3, only ingestion of STI elicits an increase of inhibitor resistant activity. This difference may be related to their effectiveness as inhibitors of midgut proteases, with STI a more potent inhibitor of FTC gut proteolytic activity *in vitro* (Fig. 6-3). Other studies have also shown that incorporating different PIs in diets elicits different responses in terms of larval digestive physiology (Broadway, 1997; Bown et al., 2004). In feeding experiments with *Helicoverpa armigera*, STI was a more effective inhibitor *in vitro* but induced a switch from inhibitor sensitive to resistant trypsin and chymotrypsin activity. By contrast, soybean Bowman-Birk inhibitor (SBBI) elicited a broad increase of trypsin and chymotrypsin activity without any qualitative change of PI-sensitivity (Bown et al., 2004). Thus, the inhibitor showing greater *in vitro* activity (STI) was less effective *in vivo* because it triggered production of resistant proteases, whereas the inhibitor with lower *in vitro* activity (SBBI) was more effective *in vivo* because no resistant proteases were produced (Bown et al., 2004). Furthermore, Bown et al. (2004) refined a compelling model analogous to mammalian digestive physiology, whereby protease secretion in the lepidopteran gut is regulated by a "monitor" peptide hormone (Jongsma and Bolter, 1997). In this model, STI binds to high affinity FTC gut receptors and triggers an adaptive switch to resistant proteases. Meanwhile the lower affinity inhibitor TI3 does not bind to gut receptors but inhibits hydrolysis of the monitor peptide resulting in hyperproduction of proteases. This model therefore provides a possible explanation why potent inhibitor activity *in vitro* does not always correspond with anti-insect activity of plant PI proteins *in vivo*.

TI3 is an effective inhibitor of FTC midgut protease activity and this inhibition translates into reduced growth upon FTC ingestion. This suggests that TI3 is an excellent

candidate for engineering pest resistance with tree biotechnology. Transgenic poplars expressing PIs have been generated with mixed results. STI expressed in *Populus nigra* does not significantly affect growth or mortality of the poplar lepidopteran pests *Lymantria dispar* or *Clostera anastomosis*, despite that protein extracts from transgenic plants inhibited midgut trypsin activity from both pests *in vitro* (Confalonieri et al., 1998). This suggests that these larvae adapt to STI, similar to the result we found for FTC reared on STI. Indeed, *Lymantria dispar* adapts to ingestion of cabbage PIs by increasing the level of PI-resistant trypsin-like activity (Broadway, 1995). By contrast, oryzacystatin expressed in hybrid aspen (*P. tremula* × *P. tremuloides*) increases mortality of the coleopteran pest *Chrysomela tremulae* (Leplé et al., 1995). The effects of TI3 ingestion should be examined for other poplar pests, including *Lymantria dispar*, whose complement of midgut proteinases has been characterized (Valaitis, 1995). The results of such studies could improve the potential value of poplar TI3 if it reduces growth of a broader range of forest pests.

7 General Discussion

The overall goal of this research was a molecular characterization of the hybrid poplar defense response. As a first step, macroarray-based expression profiling provided a global survey of the poplar wound response (Chapters 2-4). Comparative analyses were used to investigate gene expression after treatment with FTC-R and mechanical wounding, as well as to study local and systemic defense responses (Chapter 2). This macroarray analysis demonstrated that FTC-R-application induces a strong defense response in poplar leaves, which is qualitatively similar to the response induced by mechanical wounding with pliers. Gene expression patterns were similar between directly treated and systemic leaves, indicating a strong systemic response. A systemic response, albeit weaker, was also detected in poplar roots by simulated foliar herbivory, demonstrating that systemic signaling in poplar moves downward (Chapter 4). Although there was some similarity between systemic responses in leaves and roots, some distinct features were observed in the root response, most notably the apparent absence of some genes strongly expressed in leaves. The root defense response was confirmed by accumulation of KTI proteins and increased PI activity.

Among the suite of more than 100 induced genes identified by this study, several newly-identified and wound-inducible KTIs (Christopher et al., 2004) were very highly expressed after wounding and FTC-R treatments, suggesting the presence of an inducible family of poplar KTIs. This observation led to the development of the second focus of this thesis: the role played by KTIs in poplar defense and their effects on insect proteases and FTC performance (Chapters 5 and 6). Wounding of poplar leaves induced accumulation of both KTI proteins and concomitant inhibitory activity, consistent with a defensive role for the KTIs. The completed poplar genome sequence was used to study the diversity of the poplar KTI family, and five representative KTIs were selected for expression as recombinant proteins and subsequent biochemical analysis. The five selected KTI genes all encode functional protease inhibitors, but each had distinct target protease preferences. Furthermore, all five KTIs inhibited FTC midgut protease activity in *in vitro* assays. The strongest inhibitor of FTC proteases, TI3, also reduced FTC larval growth in *in vivo* assays. Moreover, TI3 ingestion elicited an increase of FTC protease activity, which is a potential adaptive response of FTC larvae to reduced midgut protease

activity. This hyperproduction of proteases may be related to the observed larval growth reduction.

In its entirety, this research has contributed to our understanding of plant defense responses and plant-pest interactions. These research contributions occur on several levels including perception of herbivore attack, the nature of inducible systemic defense responses, the functional diversity of defensive proteins and their effects on insect pests, and adaptive responses of insect pests to a plant defense protein.

7.1 Significance of this study

The finding that wounding and FTC-R can elicit a similar defense response is significant, and differs from other studies of plant-pest systems, in which insect herbivory and insect-derived elicitors induce distinct responses from wounding (Walling, 2000; Gatehouse, 2002; Reymond et al., 2004; Roda et al., 2004). This could be specific to the defense response of trees, which encounter a myriad of insect pests over their lifetime and therefore may respond with a more general defense response to pests rather than with an insect-specific response. In Chapter 3, a model for induction of plant defense responses is proposed whereby plant-based elicitors are processed and concentrated in caterpillar regurgitant. This model can explain why our severe multiple wounding treatment, predicted to release large quantities of elicitor compounds from damaged leaves, results in a wound response that is qualitatively similar to that induced by insect regurgitant. In support of this hypothesis, a mechanical caterpillar engineered to closely mimic the spatial and temporal damage caused by insect feeding, reproduced the profile of volatile emissions previously considered to be specific to insect herbivory (Mithofer et al., 2005). In addition, a recent study showed that JA accumulates in insect salivary glands (Tooker and De Moraes, 2006), suggesting that the potency of insect oral secretions is partly due to concentration of plant elicitors. Thus, my research has contributed to the development of new hypotheses regarding plant perception of insect feeding, which challenge the prevailing notion that mechanical damage is perceived differently from damage by herbivores.

The comparison of local and systemic leaf responses (Chapter 2) suggests that in poplar these responses are very similar, providing additional evidence of a strong

systemic response in poplar (Constabel et al., 2000). This firmly establishes the utility of *Populus* as a model species for studying systemic signaling. A major question that still remains to be addressed at the molecular level is systemic signaling over large distances in mature trees (see section 7.2).

In addition to most commonly described upward, acropetal movement of the defense signal, this study also showed evidence of downward, basipetal signal movement (Chapter 4). Bi-directional signaling has been reported previously for other plants in shoots (Jones et al., 1993); however, this study has demonstrated a modest systemic response can also be induced in the roots by damage to leaves. Few prior studies have examined root-shoot signaling and responses at the molecular level, and to my knowledge this is the first report of a leaf-induced defense response in roots of a tree species. A basipetal systemic defense signal may be related to a change in plant source-sink relationships. Sink strength has been suggested to drive systemic defense responses in poplar (Arnold and Schultz, 2002), and treating poplar leaves with jasmonic acid was shown to increase carbon allocation to roots (Babst et al., 2005). Rather than a defense response *per se*, the systemic root response observed here could represent a reallocation of resources away from leaf-feeding herbivores to be used for later tissue regrowth or defense responses against future herbivore attacks (Schwachtje et al., 2006). Such long-term strategies may be particularly important for trees and other long-lived perennials.

In addition to facilitating broad comparisons of inducible defense responses in poplar, the macroarray analysis generated a catalog of candidate defense genes that provided novel insights into much of poplar defense, and that are targets for future functional analyses. When this study began, none of the proteinaceous defenses of poplar had been directly shown to be detrimental to insects, although defensive roles were suspected for several inducible genes such as TIs, chitinases, and PPO. Therefore, a major goal of this research was to functionally characterize candidate genes identified from the macroarray analysis, and to ascertain whether they play a role in poplar defense. Recently, poplar PPO and chitinase were directly shown to have anti-insect properties; poplar PPO1 overexpressed in transgenic hybrid aspen (*P. tremula* × *P. alba*) significantly reduced survival and growth of FTC larvae with low vigor (Wang and Constabel, 2004a), and poplar endochitinase WIN6 retarded the development of Colorado

potato beetle when expressed in tomato plants (Lawrence and Novak, 2006). Here, I have shown that poplar TI3 is also an anti-insect defense protein, since FTC fed diets incorporating recombinant TI3 exhibited reduced growth (Chapter 6). Thus, it is now clear that proteinaceous defenses are functional and likely important components of the poplar defense response.

The analysis of the poplar KTI family also showed that sequence diversity reflects functional diversity. This finding is significant since other studies have shown that this family has undergone several duplication events followed by periods of rapid evolution, and that some TI genes show signatures of positive selection (Ingvarsson, 2005; Talyzina and Ingvarsson, 2006). Our results support a hypothesis that strong positive selection following gene duplications events has led to functional specialization of KTI genes. This has significant implications for the evolution of poplar protein defenses because the *Populus* genome contains many inducible Kunitz-type PIs but appears to contain few inducible PIs belonging to other families. Thus it appears that in poplar, the KTI family expanded rapidly through multiple duplication events, ultimately leading to specialization of individual inhibitor genes as shown in Chapter 5 and 6. This may be a special feature of a tree species, since the presence of a large and functionally diverse family of defense proteins predicts that insect pests with short-generation times will be less likely to evolve resistance to the defenses of their long-lived hosts.

7.2 Future directions

The results of this thesis provide a foundation for several follow-up studies of plant defense and tree-pest interactions. The finding that FTC-R is a potent inducer of the poplar defense response and that it contains the FAC volicitin is a starting point for additional studies of insect-derived elicitors of tree defense responses. Additional studies are needed to test synthetic FACs such as volicitin to verify that these are capable of triggering inducible poplar defenses, as has been shown for other plants (Tumlinson and Lait, 2005). Furthermore, the presence of other elicitors in FTC-R or in regurgitant of other poplar pests should also be investigated. An earlier study reported that regurgitant from gypsy moth larvae also increases pest resistance in poplar (Havill and Raffa, 1999). The pH of *Manduca sexta* regurgitant was recently suggested to shape tobacco defense

responses (von Dahl et al., 2006), and this effect of pH is also observed in poplar. Preliminary experiments with buffers of various pH values suggest that poplar leaves may also respond to the pH of FTC-R (data not shown). To detect differences among elicitors and treatments, the suite of poplar defense genes identified here (Chapter 2) and by others (Lawrence et al., 2006; Ralph et al., 2006a) can be used to compare expression patterns triggered by different elicitor components of FTC-R or by regurgitant from different poplar insect pests. With the current availability of larger cDNA microarrays enriched with herbivore-induced defense genes (Ralph et al., 2006a) and commercially-developed whole-genome poplar arrays (Jansson and Douglas, 2007), global expression profiling could be used to precisely dissect elicitor-specific effects. Understanding what additional elicitors are active in FTC-R, and how poplar responds to elicitors from different insect pests, will ultimately lead to a better understanding about how poplars adapt to herbivore stresses.

Investigation of systemic signal movement in poplar is an important avenue for further study. Given the strong systemic response in poplar saplings, a key question to address is how far the systemic response extends in larger poplar trees. Preliminary experiments indicate that the systemic signal can travel more than 1 m in poplar saplings (Yates and Constabel, unpublished data). The extensive branching and spatial distribution between local and distal tissues in poplar can be directly addressed by examing the accumulation of defense marker genes such as TI3. Such studies should also be taken beyond the controlled environments of greenhouses to investigate the inducible systemic response in mature poplar trees. Ultimately, it is important to determine whether the results observed in saplings are also found in mature trees.

In addition to studies of systemic responses in aboveground tissues, much work remains to be done on defense responses in poplar roots. Such studies should address resistance against belowground pests, or as discussed above in section 7.1 and Chapter 4, the reallocation of nutrients for later regrowth or future herbivore challenge. These hypotheses could be first tested with correlative experiments that examine the effects of induced root responses after foliar wounding and herbivory on enhanced root resistance or regrowth ability. The results of these studies may indicate whether systemic responses

in roots are important for tree defense and provide key insights into how trees manage insect challenge.

The KTIs are only one among many potential defense genes identified here, and were shown to have functions consistent with roles in herbivore defense. TI3 in particular was also shown to have direct detrimental effects on insect growth. In addition to these KTIs, there are many other candidate genes that may have functions in defense and should be tested. For example, an apyrase and acid phosphatase are very strongly induced by wounding and FTC-R treatment (Chapter 2). Since recent studies showed that amino acid hydrolases (threonine deaminase, arginase) exert detrimental effects on insect herbivores by degrading essential amino acids in the insect gut, these two phosphate metabolism enzymes could also potentially have antinutritive effects by disrupting uptake of phosphate-containing compounds. In support of a direct defensive role for acid phosphatase, a recent study showed that an *Arabidopsis* protein with acid phosphatase activity had detrimental effects on insects when fed in artificial diets. Furthermore, these effects were dependent upon the acid phosphatase activity (Liu et al., 2005). Several other candidate genes have wound- and FTC-R-inducible expression patterns suggesting anti-herbivore effects, and should be investigated further. Using this strategy for genes hypothesized to encode products that directly affect insect performance, insect bioassays could be used to test the effect of the gene product generated as a recombinant proteins (Chapter 5 and 6) or via transgenic or transfection approaches (Wang and Constabel, 2004a; Lawrence and Novak, 2006). However, there may be some limitations to this approach depending on the gene to be studied. For example, if the gene encodes an enzyme that requires specific substrates for anti-insect activity, these approaches will be limited by substrate availability. This could also occur if the enzyme and substrate are compartmentalized within plant cells or tissues. Furthermore, ectopic expression of the gene product could divert substrates away from key biochemical pathways and cause disruptive effects. Alternatively, some plant defenses may be effective against some insect species but not others. For example, I found that poplar KTIs were effective inhibitors of *Malacosom disstria* proteases but not those of *Mamestra configurata*. Thus, when testing the potential anti-insect activity of a gene product, careful consideration

should be made to ensure that the insect bioassays do not produce misleading results. This may entail testing the candidate defense gene with multiple approaches.

Since TI3 is an effective inhibitor of insect protease activity and insect performance (Chapter 5 and 6), it should be tested with transgenic plants, especially trees, for enhanced pest resistance. Although transgenic poplars expressing PIs have been generated with mixed results, hybrid aspen (*P. tremula* × *P. tremuloides*) expressing rice oryzacystatin has enhanced resistance against poplar leaf beetle (*Chrysomela tremulae*) (Leplé et al., 1995). The use of TI3 may be very desirable for poplar biotechnology since it would not involve expression of a foreign gene. In addition, the anti-herbivore effects of TI3 should be examined for additional poplar pests, such as gypsy moth (*Lymantria dispar*). If TI3 is an effective defense protein against a broader range of pests, its value would increase as a target for tree biotechnology. Based on the strong effects with TI3, a key future research direction is to examine other poplar recombinant TIs for direct effects against poplar pests. For example, poplar TI2 is a very effective inhibitor of FTC midgut protease activity, but inhibits a different profile of proteases (Chapter 5) and therefore may have distinct effects *in vivo*. In addition, it would be interesting to test the effects of TI4, TI5 and TI6 against other poplar pests, since their functional diversity predicts that they should have detrimental effects against different pests. Since purified recombinant proteins have been generated for all of these proteins, testing their effects against poplar pests requires only scaling-up their production for insect bioassays. Finally, the functional and biochemical properties of recombinant proteins from clade C of the poplar KTI family should be examined, since none of these were studied as part of this research. These TIs appear to be induced by pathogen infection rather than wounding and should therefore also be tested for antimicrobial activity with growth inhibition assays. Some KTIs from other plants are effective antimicrobial proteins against plant pathogens (Macedo et al., 2004; Kim et al., 2005). If the KTIs from clade C are antimicrobial and predicted to play a role in pathogen defense, this would represent an interesting dichotomy of the poplar KTI family between defense against herbivores and pathogens.

Another direction for future research is the analysis of co-evolution between the poplar KTI genes and insect protease genes. This is especially interesting because unlike other plant-pest molecular interactions, plant PIs bind their target pest proteases, thereby

facilitating studies of co-evolution since the gene products from plant and insect interact directly. Considering the rapid evolution of the poplar KTI family, it is reasonable to predict that the insect protease genes are rapidly evolving as well, since some insects secrete PI-resistant proteases (Broadway, 1995; Volpicella et al., 2006). Previous studies investigating the molecular evolution of plant PIs and insect proteases and have found that the amino acid residues of PIs and proteases that interact directly are hypervariable, suggesting positive selection for these residues and co-evolution between PIs and proteases (Lopes et al., 2004). Studies of molecular evolution of the KTI family have likewise identified amino acid residues that are hypervariable and under positive selection (Talyzina and Ingvarsson, 2006); however, unlike other plant PIs, these hypervariable residues do not appear to interact directly with target proteases (data not shown). A first step toward studying TI-protease co-evolution would be to isolate and sequence the proteases from FTC guts that are sensitive or resistant to poplar KTIs. Knowing which proteases are sensitive and resistant to KTIs will permit a detailed analysis of the residues that are important for the interaction between the inhibitor and protease. Moreover, a small-scale EST sequencing project of larval midguts would generate a database of gene sequences encoding expressed proteases. Studying the co-evolution of poplar KTIs and proteases of poplar pests such as FTC presents an exciting opportunity; both *Populus* and *Malacosoma* species occur naturally throughout the Northern hemisphere (Fitzgerald, 1995), providing an evolutionary resource of numerous plant-pest populations that have not been influenced by human intervention (e.g. artificial selection).

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9 Appendix 1: Supplemental Data

The following pages contain the Supplementary Tables and Figures referred to in Chapters 2, 4, 5, and 6. A Table of Contents for the Supplemental Data is printed on preliminary page x.

Supplemental Table 2-1. Mean expression ratios for all significantly induced or repressed genes.

Only the first page of this table is printed. The entire table is available online on the New Phytologist website at: http://www.blackwell-synergy.com/doi/suppl/10.1111/j.1469-8137.2006.01877.x

Mean expression ratio after challenge with mechanical wounding (pliers) or forest tent caterpillar (*Malacosoma disstria*) regurgitant. Only genes up- or down-regulated twofold with $P < 0.05$ are shown. Plants were challenged three times at 1 h intervals, and were harvested 24 h after the initial treatment.
Local (treated) leaves and systemic (untreated leaves on treated plants) leaves are compared to corresponding leaves of control (unwounded, wounding; mock water treated, FTC regurgitant) plants.
Student *t*-test (paired, one tailed) was perfomed on the log-transformed data from 3 independent control vs treatment experiments.

Plier wounding local Mean (n=3)	*P*-value	**Plier wounding systemic** Mean (n=3)	*P*-value	**FTC regurgitant local** Mean (n=3)	*P*-value	**FTC regurgitant systemic** Mean (n=3)	*P*-value	**NCBI accessio**	**EST numbe**	**Putative function**
Genes up-regulated at least twofold:										
261.026	0.00576	57.878	0.00053	12.745	0.00363	12.813	0.05376	CN192741	H1506	Poplar endochitinase *win6.2C*
161.361	0.00138	59.861	0.00576	3.617	0.01082	7.770	0.19488	CN192556	H1078	Poplar endochitinase *win6.2C*
152.210	0.01099	52.664	0.01711	6.802	0.04037	9.859	0.10383	CN192595	H1141	Poplar endochitinase *win8*
144.985	0.00378	33.320	0.00778	4.960	0.00261	5.503	0.08615	CN193334	H831	polyphenol oxidase
114.182	0.00503	16.190	0.00718	3.063	0.09798	2.363	0.45364	CN193330	H828	Kunitz trypsin inhibitor *PtdTI4*
97.792	0.00283	30.912	0.00503	9.568	0.00261	9.131	0.06104	CN192786	H1644	lipase, class 3
90.001	0.00611	34.618	0.00051	7.389	0.01265	11.929	0.03966		F-64	apyrase
69.001	0.01268	10.165	0.00155	4.572	0.07802	5.553	0.02972	AF263611	PPO1	polyphenol oxidase *PtdPPO1*
67.733	0.01582	13.280	0.01747	6.578	0.11611	2.358	0.05459	CN192805	H1685	Kunitz trypsin inhibitor *PtdTI5*
55.405	0.00520	39.815	0.00914	8.209	0.00137	11.346	0.04731	CN192930	H1949	Poplar vegetative storage protein *win4.5*
55.003	0.00325	33.287	0.00504	6.839	0.00956	9.690	0.02193	CN193208	H64	apyrase
33.405	0.00221	23.859	0.00491	6.855	0.01116	7.564	0.06790	CN192936	H1958	unknown protein
32.079	0.00818	25.730	0.00119	3.292	0.00071	3.883	0.09151	CN192736	H1496	Kunitz trypsin inhibitor *PtdTI3*
30.355	0.00114	7.925	0.00334	1.768	0.16707	2.216	0.05032	CN193425	H950	Poplar vegetative storage protein *pni288*
29.672	0.00734	29.111	0.00274	3.421	0.03383	4.731	0.16438	CN192549	H1059	Kunitz trypsin inhibitor *PtdTI3*
25.524	0.04660	22.806	0.01614	3.930	0.28516	7.516	0.07659	CN192663	H1286	acyl-activating enzyme
25.223	0.00240	13.250	0.00228	5.091	0.01175	6.108	0.03362		F-108	acid phosphatase, class B
23.888	0.00966	13.431	0.01786	4.870	0.00104	4.810	0.00266		F-13	Poplar *Pop3 / SP1*
22.722	0.00254	13.502	0.00086	6.665	0.02047	5.768	0.02455	CN193016	H244	acid phosphatase, class B
21.676	0.00176	10.629	0.00103	3.397	0.04082	4.307	0.04765	CN192760	H1594	beta-amylase
18.914	0.04592	11.854	0.00901	3.285	0.13547	6.343	0.08525	CN192663	H1286	acyl-activating enzyme
17.528	0.02121	11.074	0.00037	2.550	0.02043	2.151	0.09932	CN193274	H762	cytochrome P450
17.158	0.00777	12.271	0.00063	5.496	0.02849	6.456	0.04062		H268	*Pop3- / SP1*-like

Supplemental Table 2-2. Mean expression ratios for all genes represented on macroarray.

Only the first page of this table is printed. The entire table is available online on the New Phytologist website at: http://www.blackwell-synergy.com/doi/suppl/10.1111/j.1469-8137.2006.01877.x

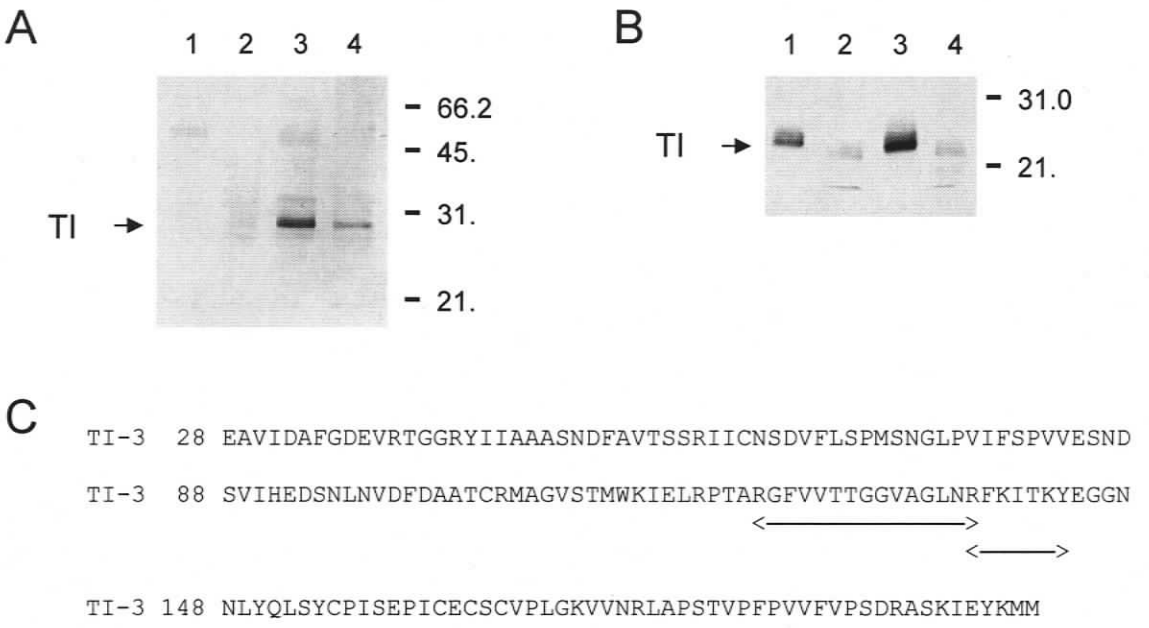
Mean expression ratio after challenge with mechanical wounding (pliers) or forest tent caterpillar (*Malacosoma disstria*) regurgitant. Shown are all cDNAs spotted on the array. Plants were challenged three times at 1 h intervals, and were harvested 24 h after the initial treatment.

Local (treated) leaves and systemic (untreated leaves on treated plants) leaves are compared to corresponding leaves of control (unwounded, wounding; mock water treated, FTC regurgitant) plants.

Student *t*-test (paired, one tailed) was perfomed on the log-transformed data from 3 independent control vs treatment experiments.

FDR (*Q*-value) was calculated using the R statistical package (http://www.r-project.org/) as described by Storey and Tibshirani, 2003.

Plier wounding local			**Plier wounding systemic**			**FTC regurgitant local**			**FTC regurgitant systemic**			**NCBI accession**	**EST number**	**Putative function**
Mean (n=3)	*P*-value	*Q*-value	Mean (n=3)	*P*-value	*Q*-value	Mean (n=3)	*P*-value	*Q*-value	Mean (n=3)	*P*-value	*Q*-value			
3.671	0.0034	0.0486	4.428	0.0002	0.0271	0.739	0.0335	0.2929	1.321	0.3960	0.6026	CN192896	H1869	alcohol acyl transferase
1.605	0.0630	0.1143	1.844	0.0244	0.1314	0.850	0.1736	0.3744	1.169	0.1449	0.4557	CN192666	H1293	1-aminocyclopropane-1-carboxylate oxidase
0.659	0.0354	0.0905	0.777	0.0834	0.2747	0.781	0.1062	0.3548	0.894	0.2023	0.4963	CN192954	H201	chlorophyll a/b binding protein
0.702	0.0714	0.1250	0.791	0.0019	0.0477	1.067	0.3861	0.4526	1.052	0.0284	0.4431	CN193057	H351	oxygen evolving enhancer protein 3
1.171	0.0541	0.1047	0.923	0.0100	0.0839	1.143	0.3400	0.4407	0.929	0.1337	0.4527	CN192848	H1763	fructose-1,6-bisphosphatase
3.519	0.0069	0.0545	2.505	0.0230	0.1268	1.386	0.0685	0.3391	1.541	0.1105	0.4431	CN192573	H1102	glycerophosphodiester phosphodiesterase
1.813	0.0317	0.0873	1.393	0.1035	0.3063	0.869	0.2794	0.4227	1.153	0.1983	0.4963	CN193115	H523	arginine decarboxylase
1.219	0.0729	0.1257	0.948	0.3406	0.4659	1.430	0.1984	0.3885	1.087	0.1554	0.4685	CN192920	H1928	NADH-ubiquinone oxidoreductase 75 kDa subunit
0.498	0.0921	0.1429	1.191	0.4813	0.5538	1.897	0.4608	0.4736	1.141	0.4856	0.6468	CN193075	H445	actin depolymerizing factor
0.707	0.0765	0.1283	0.680	0.1204	0.3200	1.017	0.3699	0.4526	1.016	0.4590	0.6360	CN193246	H717	auxin-regulated protein
1.149	0.2794	0.2751	1.765	0.3761	0.4912	0.880	0.2692	0.4166	1.042	0.4993	0.6531	CN192688	H1331	NAC domain protein
0.610	0.0769	0.1283	0.475	0.0081	0.0793	1.147	0.4306	0.4647	1.010	0.4257	0.6225	CN193331	H829	expressed protein [Arabidopsis thaliana]
1.200	0.1474	0.1881	1.124	0.4531	0.5394	0.922	0.3156	0.4343	1.018	0.4549	0.6343	CN193033	H28	unknown [Arabidopsis thaliana]
0.579	0.0403	0.0957	0.596	0.0811	0.2709	0.880	0.2845	0.4232	0.867	0.1386	0.4546	CN193317	H813	expressed protein [Arabidopsis thaliana]
0.749	0.0326	0.0883	0.631	0.0951	0.2920	1.246	0.3161	0.4343	1.301	0.3532	0.5744	CN192579	H1115	no hits
0.888	0.2366	0.2494	1.210	0.4871	0.5559	1.160	0.4996	0.4922	1.309	0.2658	0.5286	CN193110	H518	no hits
0.975	0.3765	0.3290	1.359	0.2252	0.3830	1.071	0.2539	0.4099	1.229	0.1287	0.4527	CN192593	H1139	UDP-glucuronosyl and UDP-glucosyl transferase
2.333	0.0006	0.0477	1.642	0.0389	0.1723	1.334	0.3349	0.4407	1.744	0.1408	0.4554	CN192858	H178	enolase
7.773	0.0041	0.0486	5.905	0.0000	0.0099	1.584	0.1552	0.3695	2.335	0.0909	0.4431	CN193183	H605	prephenate dehydratase
0.917	0.2240	0.2433	0.736	0.0840	0.2749	0.867	0.2342	0.3983	1.300	0.1093	0.4431	CN192622	H120	unknown [Euphorbia esula]
1.083	0.2853	0.2776	1.166	0.3677	0.4867	1.294	0.0246	0.2653	1.322	0.1789	0.4876	CN193036	H289	WD-repeat protein GhTTG2
1.576	0.0174	0.0685	1.134	0.2497	0.4035	0.909	0.2592	0.4127	1.137	0.0151	0.4431	CN193388	H9	S-like RNase
0.881	0.2510	0.2578	0.794	0.1959	0.3707	1.536	0.0828	0.3440	1.287	0.3940	0.6025	CN193301	H794	DnaJ protein family
0.974	0.3888	0.3379	0.871	0.1758	0.3614	0.912	0.1399	0.3695	1.318	0.2144	0.5034	CN192829	H1712	plastid protein [Arabidopsis thaliana]



Supplemental Figure 5-1. Analysis of TI protein stability after ingestion by forest tent caterpillar (FTC).

Protein isolated from frass of FTC fed control or MeJA-sprayed foliage was analyzed by western blots (**A**, **B**) and LC-MS/MS (**C**).

A and B. Western blots of TI2 (**A**) and TI3 (**B**) protein accumulation. Hybrid poplar foliage was collected from untreated, control trees (lane 1) or trees sprayed with methyl-jasmonate (MJ; lane 3), and leaves were fed to FTC. Frass was collected from FTC fed control foliage (lane 2) or MeJA-sprayed foliage (lane 4).

[Western analysis carried out by Nicole Dafoe, University of Victoria.]

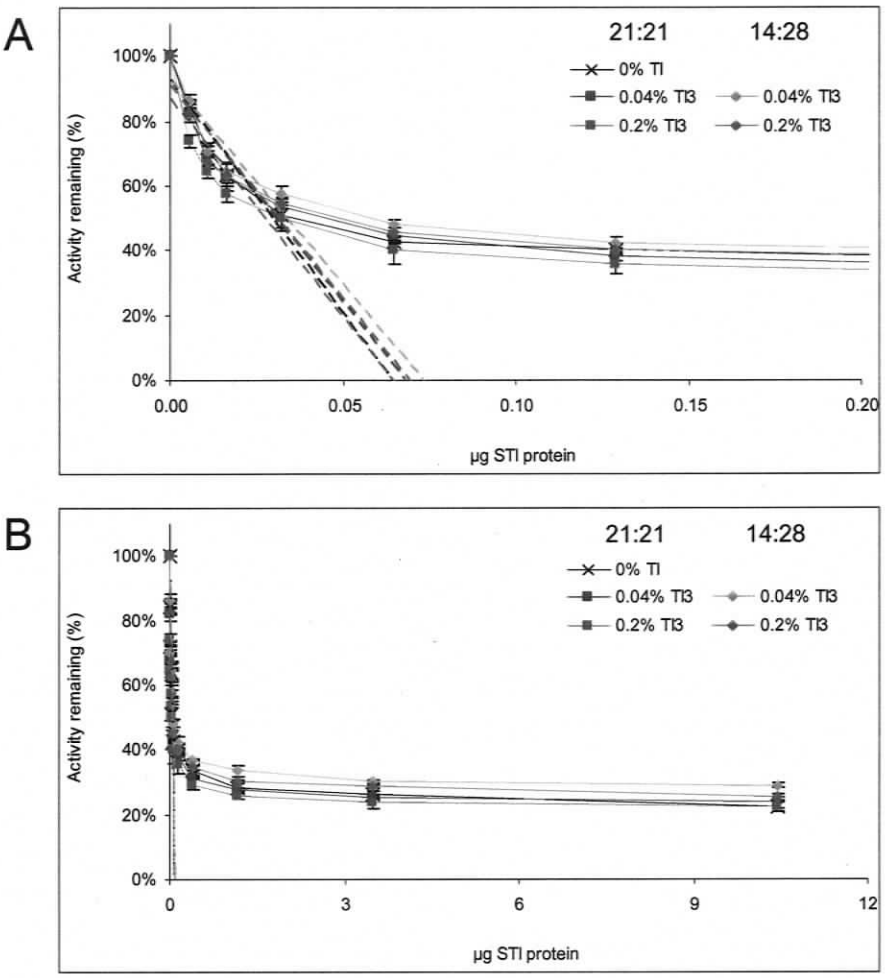
C. Amino acid sequence of TI3 (without signal peptide) with peptides obtained by LC-MS/MS.

[LC-MS/MS analysis of frass carried out by Dr. Curtis Wilkerson and Dr. Gregg Howe, Michigan State University, USA.

Supplemental Table 6-1. Effect of ingested plant TIs on protease composition of midgut extracts from *Malacosoma disstria* larvae as determined by commercial protease inhibitors[a].

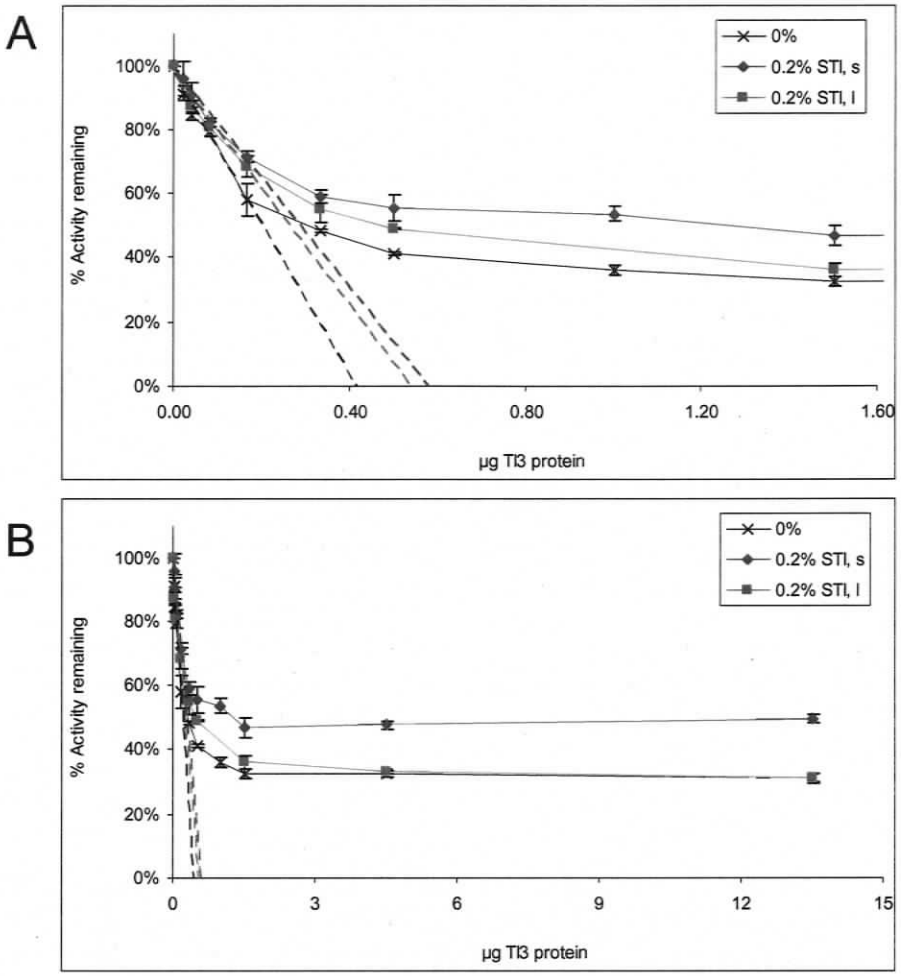
		Inhibition (%) ± SE					
		21:21 diet				14:28 diet	
Protease inhibitor		none	0.2% STI	0.04% TI3	0.2% TI3	0.04% TI3	0.2% TI3
		pH 9.5					
PMSF	10 mM	94.3 ± 3.1	95.6 ± 0.3	96.7 ± 1.9	98.4 ± 1.1	95.0 ± 5.1	98.6 ± 2.6
	25 mM	100.7 ± 2.2	97.7 ± 1.4	98.6 ± 1.9	100.2 ± 1.7	102.3 ± 0.7	98.6 ± 3.4
TLCK	1 mM	38.1 ± 2.4	24.3 ± 0.6	26.0 ± 0.2	27.4 ± 1.3	33.1 ± 2.3	32.6 ± 2.0
	10 mM	92.7 ± 4.0	93.3 ± 3.2	88.5 ± 2.6	85.2 ± 3.1	89.9 ± 2.8	86.4 ± 5.9
TPCK	1 mM	17.3 ± 1.6	18.6 ± 2.0	21.5 ± 1.6	16.2 ± 0.9	16.4 ± 5.5	24.1 ± 3.7
IAA	1 mM	0.7 ± 5.0	n.d.	1.0 ± 2.9	7.9 ± 5.2	4.3 ± 1.9	-1.0 ± 3.0
Pepstatin	10 µM	8.8 ± 3.4	n.d.	1.4 ± 3.4	2.5 ± 3.8	2.8 ± 0.9	2.8 ± 4.0
EDTA	50 mM	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
		pH 10.5					
PMSF	10 mM	66.6 ± 2.0	58.2 ± 0.5	64.0 ± 4.3	61.0 ± 2.3	78.7 ± 1.3	76.9 ± 3.0
	25 mM	96.6 ± 0.5	96.9 ± 0.6	95.0 ± 1.2	95.2 ± 1.2	98.1 ± 1.0	98.0 ± 2.1
TLCK	1 mM	48.9 ± 2.0	28.4 ± 1.3	47.7 ± 1.1	47.4 ± 1.2	49.3 ± 0.9	52.6 ± 0.6
	10 mM	76.7 ± 0.5	73.0 ± 1.0	73.7 ± 1.9	74.5 ± 0.9	72.4 ± 1.6	71.5 ± 0.5
TPCK	1 mM	12.5 ± 1.7	20.9 ± 1.5	9.4 ± 3.3	8.6 ± 2.0	5.9 ± 1.7	12.4 ± 0.9
IAA	1 mM	7.3 ± 2.6	n.d.	9.1 ± 1.5	11.1 ± 1.8	13.9 ± 1.8	8.7 ± 1.2
Pepstatin	10 µM	10.5 ± 3.1	n.d.	7.8 ± 1.7	9.3 ± 1.4	10.5 ± 1.5	7.0 ± 1.6
EDTA	50 mM	19.0 ± 1.8	n.d.	9.5 ± 0.5	10.0 ± 2.6	7.4 ± 2.5	7.2 ± 0.4
		pH 11.5					
PMSF	10 mM	60.6 ± 2.0	57.3 ± 1.9	60.8 ± 4.8	56.6 ± 3.4	67.1 ± 6.2	63.2 ± 5.3
	25 mM	92.9 ± 1.9	91.4 ± 2.1	89.3 ± 2.0	89.6 ± 1.8	95.2 ± 0.9	89.4 ± 2.5
TLCK	1 mM	60.1 ± 0.9	39.5 ± 1.4	61.8 ± 0.9	60.6 ± 0.6	63.5 ± 0.5	62.4 ± 0.4
	10 mM	79.2 ± 0.6	72.7 ± 0.8	75.7 ± 0.3	76.7 ± 0.8	74.9 ± 0.8	74.2 ± 0.3
TPCK	1 mM	17.0 ± 2.7	26.6 ± 4.4	11.6 ± 0.8	16.4 ± 0.5	8.8 ± 2.3	12.4 ± 4.0
IAA	1 mM	14.6 ± 2.6	n.d.	10.5 ± 4.2	14.1 ± 0.4	15.9 ± 1.9	13.2 ± 0.7
Pepstatin	10 µM	0.2 ± 3.8	n.d.	1.8 ± 6.3	4.0 ± 0.4	5.8 ± 1.6	12.6 ± 4.8
EDTA	50 mM	29.0 ± 0.6	n.d.	25.4 ± 3.2	25.0 ± 0.7	20.4 ± 1.4	21.6 ± 1.4

[a] FTC larvae were fed artificial diets for two protein levels (21% protein : 21% carbohydrate, 14% protein : 28% carbohydrate), and supplemented with 0%, 0.04% and 0.2% soybean TI (STI) and poplar TI3. Total proteolytic activity of crude midgut extracts was assayed by asocasein hydrolysis with inhibitor added to the final concentration shown and determined for the pH range 9.5 - 11.5. Inhibition (%) is determined from activity without inhibitor added. Standard error is calculated from three replicate assays. n.d. = not determined



Supplemental Figure 6-1. Effect of ingestion of poplar TI3 on levels of soybean TI (STI)-resistant protease activity in midguts of *Malacosoma disstria* larvae from TI3 feeding experiments.

Larvae were fed protein-rich (21:21) and protein-poor (14:28) diets incorporating 0.04% or 0.2% TI3, and STI-resistant protease activity was determined by titration of STI with midgut extracts, followed by azocasein hydrolysis at pH 11.5. STI inhibition is plotted on small- (**A**) and large-scale abscissae (**B**) to visualize the similar inhibition profiles and maxima of larval proteases fed diet with and without TI3. Error bars are standard error of three technical replicates. Maximum protease inhibition and the half-concentration of STI required to achieve maximum inhibition were determined from these *in vitro* assays and are shown in Fig. 6-6C.



Supplemental Figure 6-2. Effect of ingestion of soybean TI (STI) on levels of TI3-resistant protease activity in midguts of *Malacosoma disstria* larvae from STI feeding experiments.

Larvae were fed protein-rich diet incorporating 0.2% STI, and TI3-resistant protease activity was determined by titration of poplar TI3 with midgut extracts, followed by azocasein hydrolysis at pH 11.5. TI3 inhibition is plotted on small- (**A**) and large-scale abscissae (**B**) to visualize the similar inhibition profiles yet different inhibition maxima of larvae fed diet with 0.2% STI with normal growth (large, L) and reduced growth (small, S) compared with larvae fed diet without STI. Error bars are standard error of three technical replicates. Maximum protease inhibition and the half-concentration of TI3 required to achieve maximum inhibition were determined from these *in vitro* assays and are shown in Fig. 6-7C.